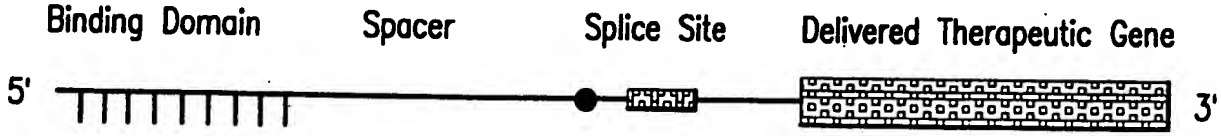

Binding Domain
Spacer
Splice Site
Delivered Therapeutic Gene
5'
3'

FIG.1A

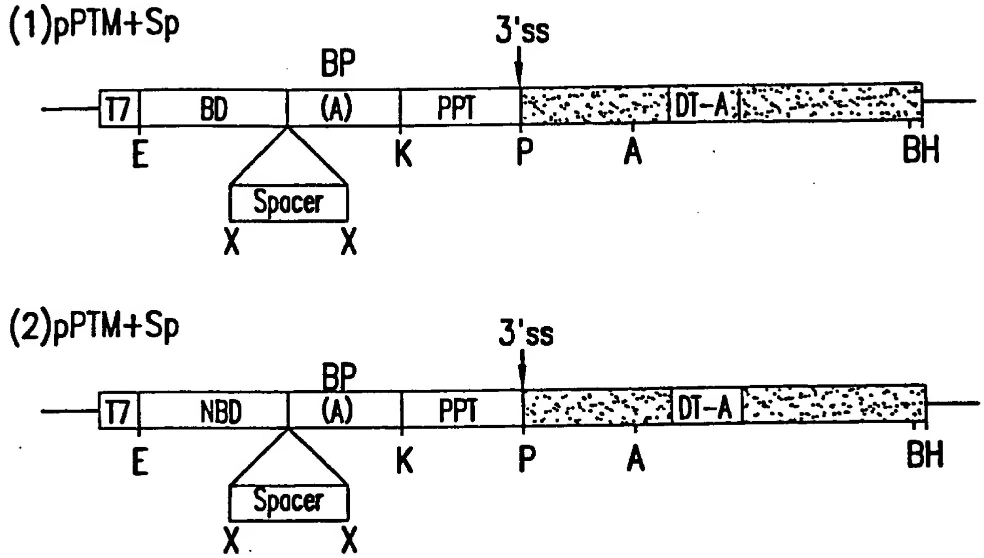

(1)pPTM+Sp
3'ss
BP
T7
BD
(A)
PPT
DT-A
E
K
P
A
BH
Spacer
X
X
(2)pPTM+Sp
3'ss
BP
T7
NBD
(A)
PPT
DT-A
E
K
P
A
BH
Spacer
X
X

FIG.1B

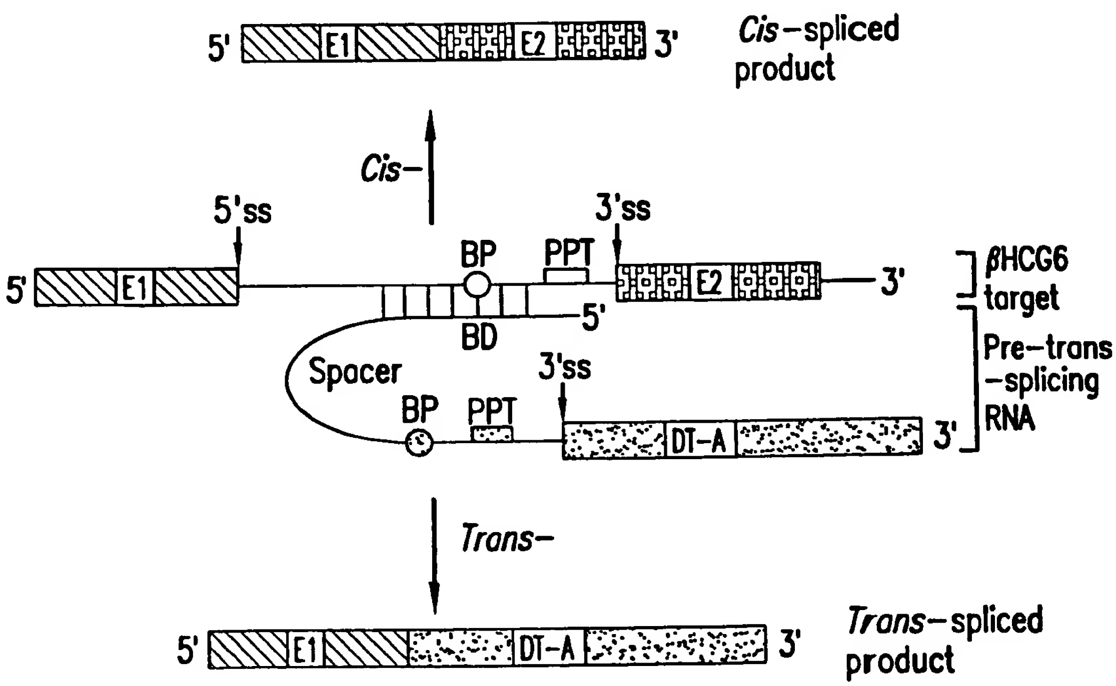

5'
E1
E2
3'
Cis–spliced product
Cis–
5'ss
3'ss
BP
PPT
E1
E2
3'
βHCG6 target
BD
5'
Pre–trans –splicing RNA
Spacer
3'ss
BP
PPT
DT-A
3'
Trans–
5'
E1
DT-A
3'
Trans–spliced product

FIG.1C


PTM RNA
Time (min)
PTM+Sp
PTM-Sp
Py(-)AG(-)
BP(-)Py(-)AG(-)
Marker (bp)
0 60 0 60 0 60 0 60
Trans-spliced 466bp product
600
500
400
300
1 2 3 4 5 6 7 8

FIG.2A

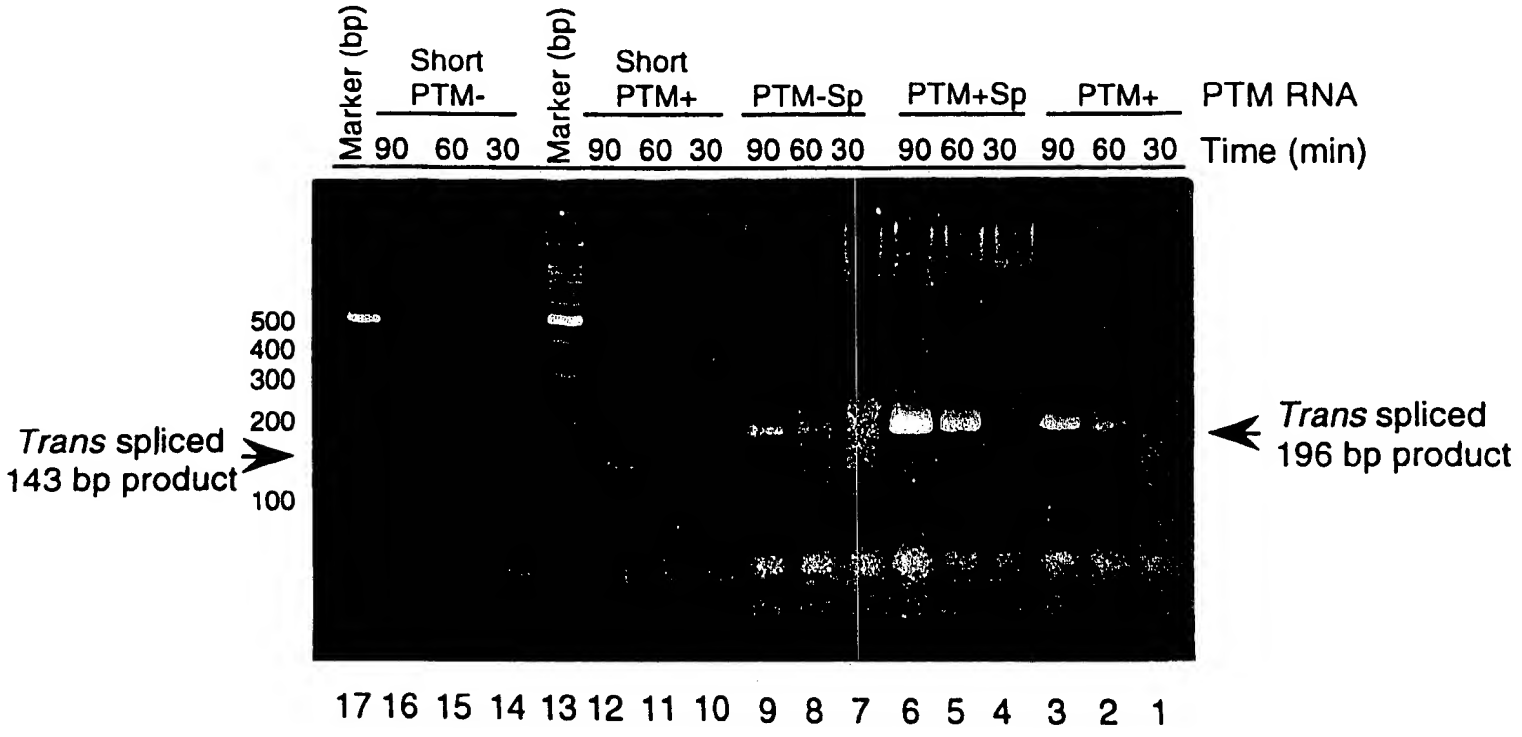


FIG.2B

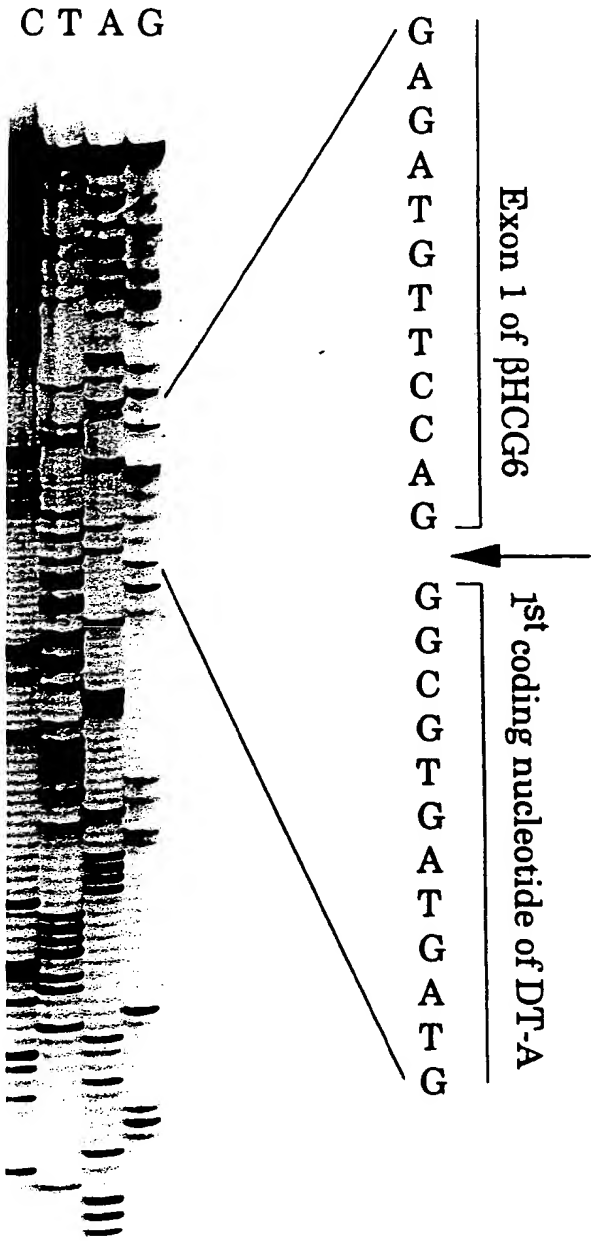


FIG.3

FIG.4A

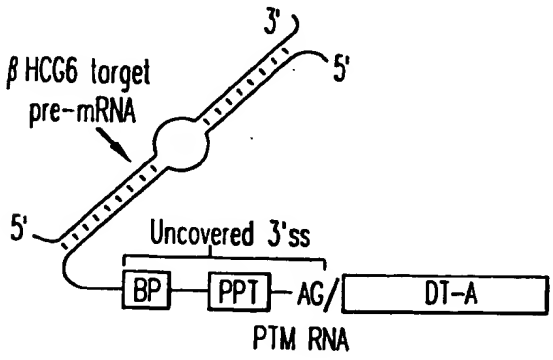


FIG.4B

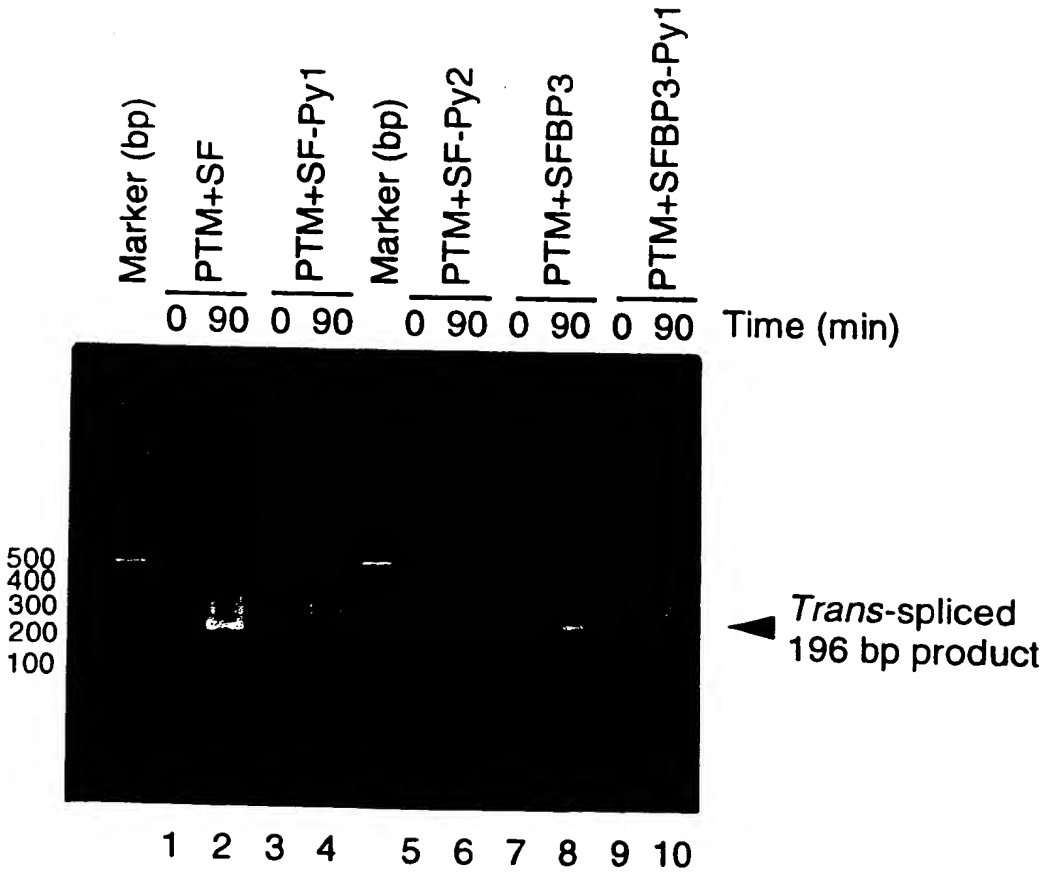


FIG.4C

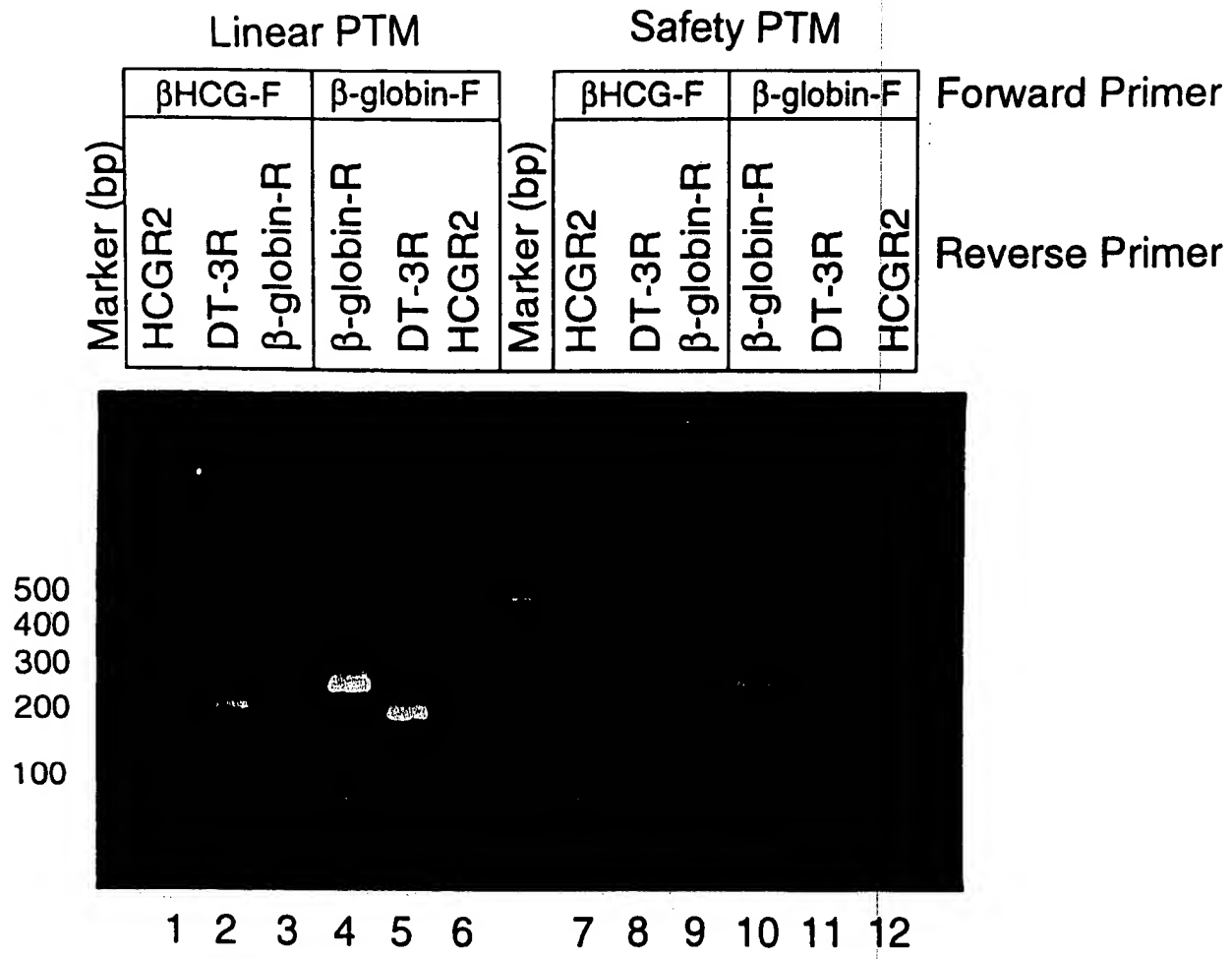

Linear PTM
Safety PTM
βHCG-F
β-globin-F
βHCG-F
β-globin-F
Forward Primer
Marker (bp)
HCGR2
DT-3R
β-globin-R
β-globin-R
DT-3R
HCGR2
Marker (bp)
HCGR2
DT-3R
β-globin-R
β-globin-R
DT-3R
HCGR2
Reverse Primer
500
400
300
200
100
1 2 3 4 5 6 7 8 9 10 11 12

FIG.5

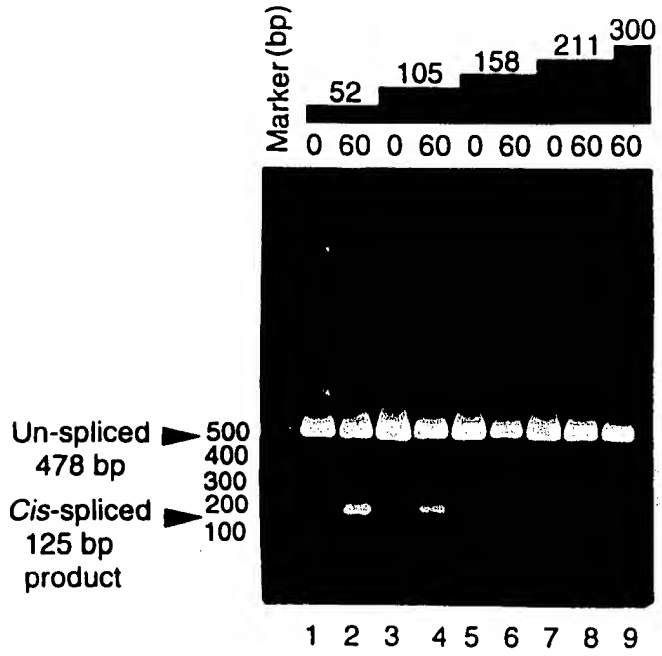


FIG.6A

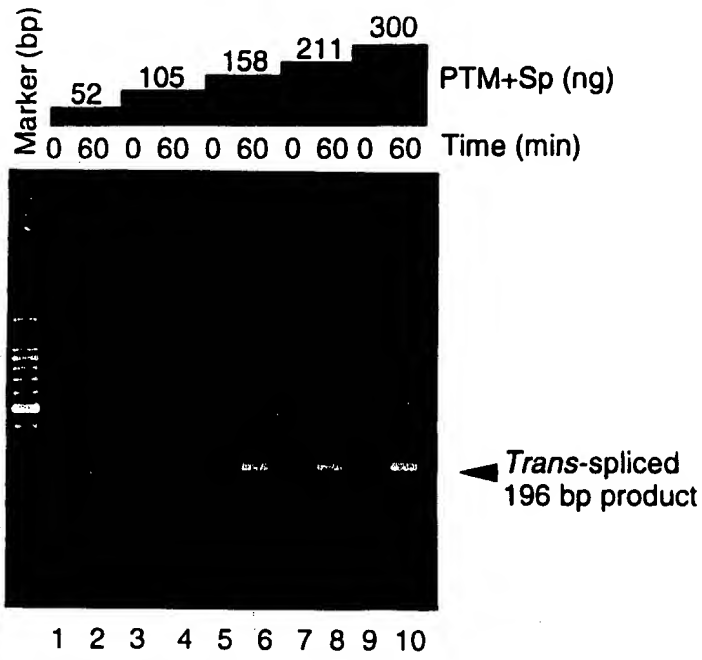


FIG.6B

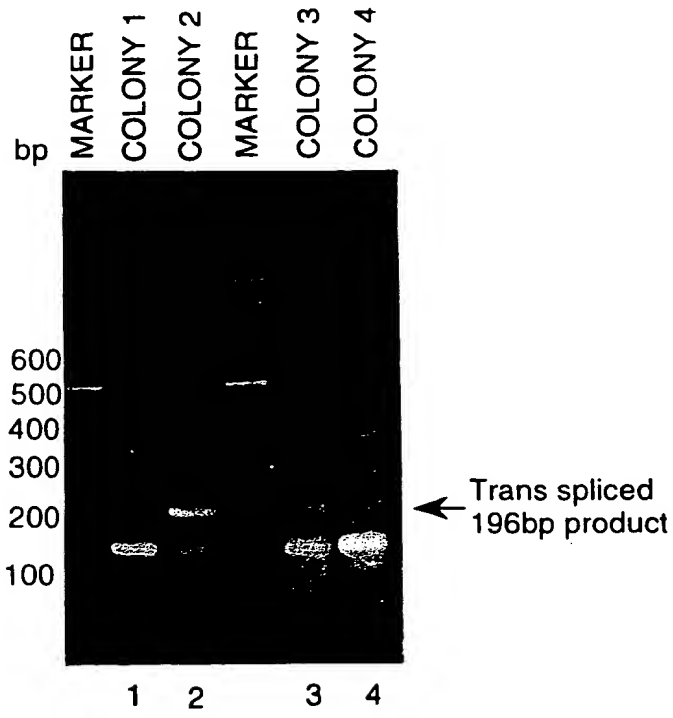


FIG.7A

EXON 1 OF βHCG6

5'-CAGGGGACGCACCAAGGATGGAGATGTTCCAG-GGCGCTGATGATGTTGTT

1ST CODING NUCLEOTIDE OF DT-A

GATTCTTCTTAAATCTTTTGTGATGGAAAACTTTTCTTCGTACCACGGGACTA

AACCTGGTTATGTAGATTCCATTCAAAAA-3'

FIG.7B

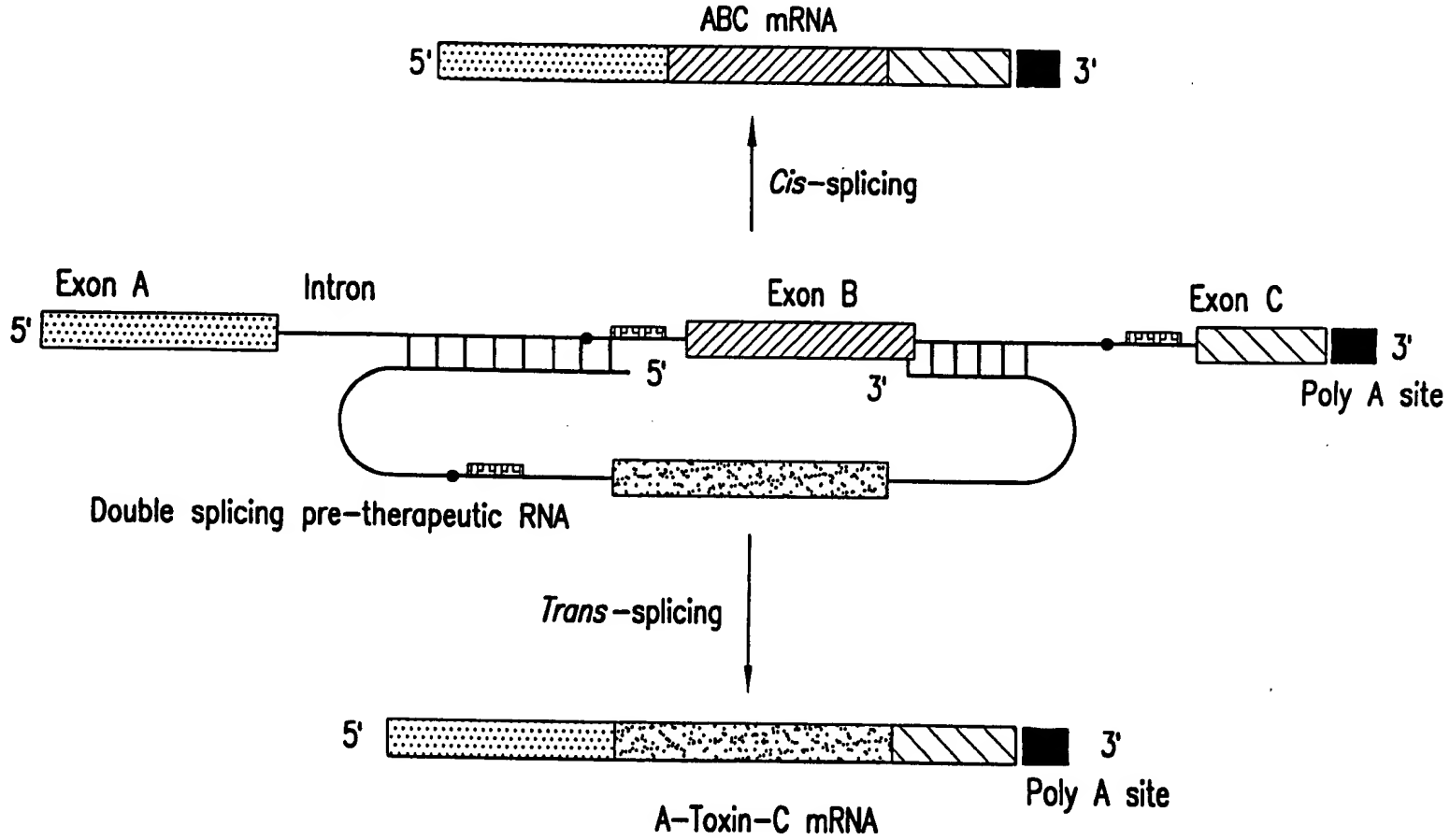
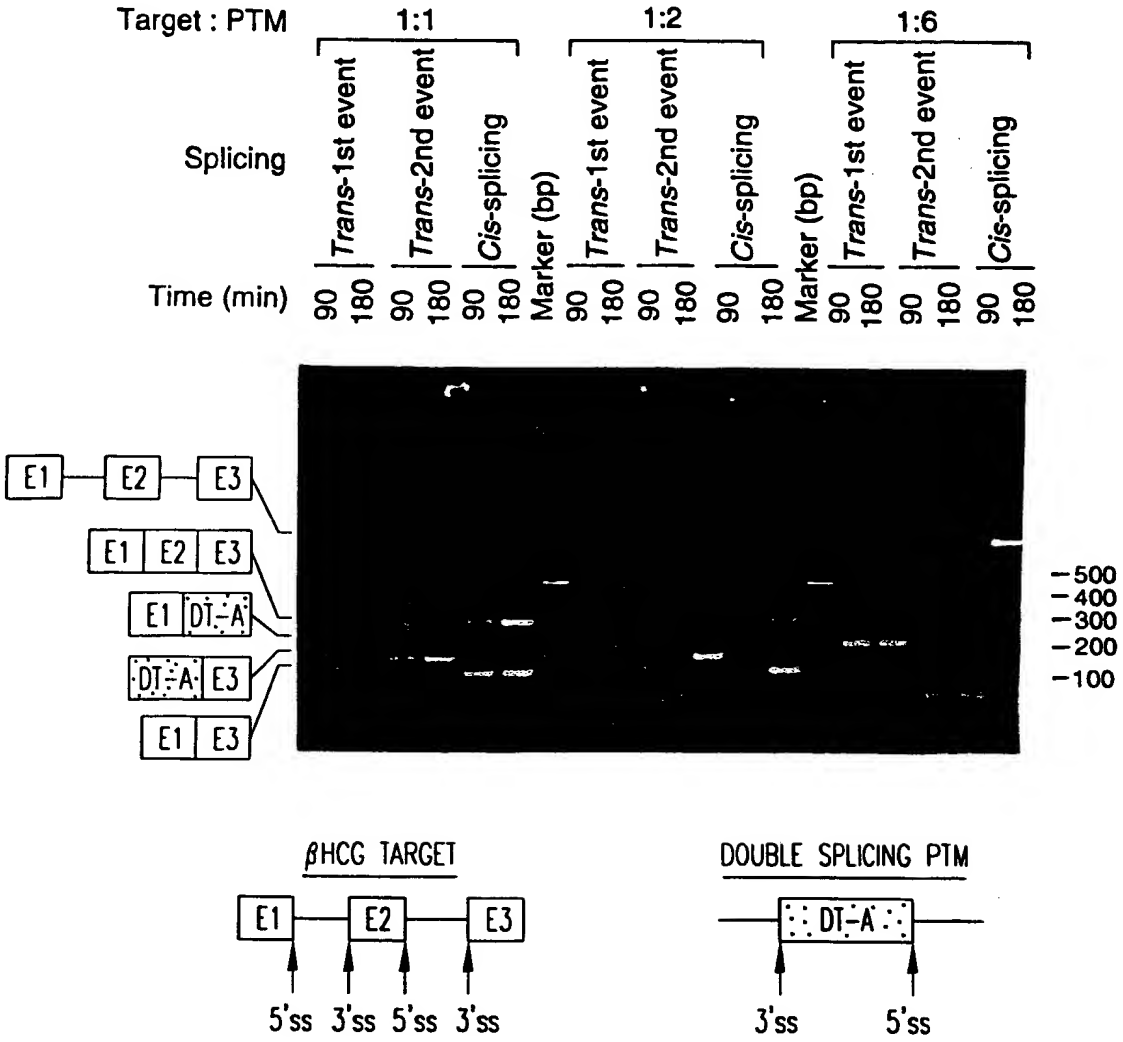

ABC mRNA
5'
3'
Cis–splicing
Exon A
Intron
Exon B
Exon C
5'
5'
3'
3'
Poly A site
Double splicing pre–therapeutic RNA
Trans–splicing
5'
3'
Poly A site
A–Toxin–C mRNA

FIG.8A



Cis- spliced products

E1 E2 E3 = NORMAL *cis*- SPLICING (277bp)

E1 E3 = Exon SKIPPING (110bp)

Trans- spliced products

E1 DT-A = 1st EVENT, 196bp. *Trans*- SPLICING BETWEEN 5' ss OF TARGET & 3' ss OF PTM.

DT-A E3 = 2nd EVENT, 161bp. *Trans*- SPLICING BETWEEN 3' ss OF TARGET & 5' ss OF PTM.

FIG.8B

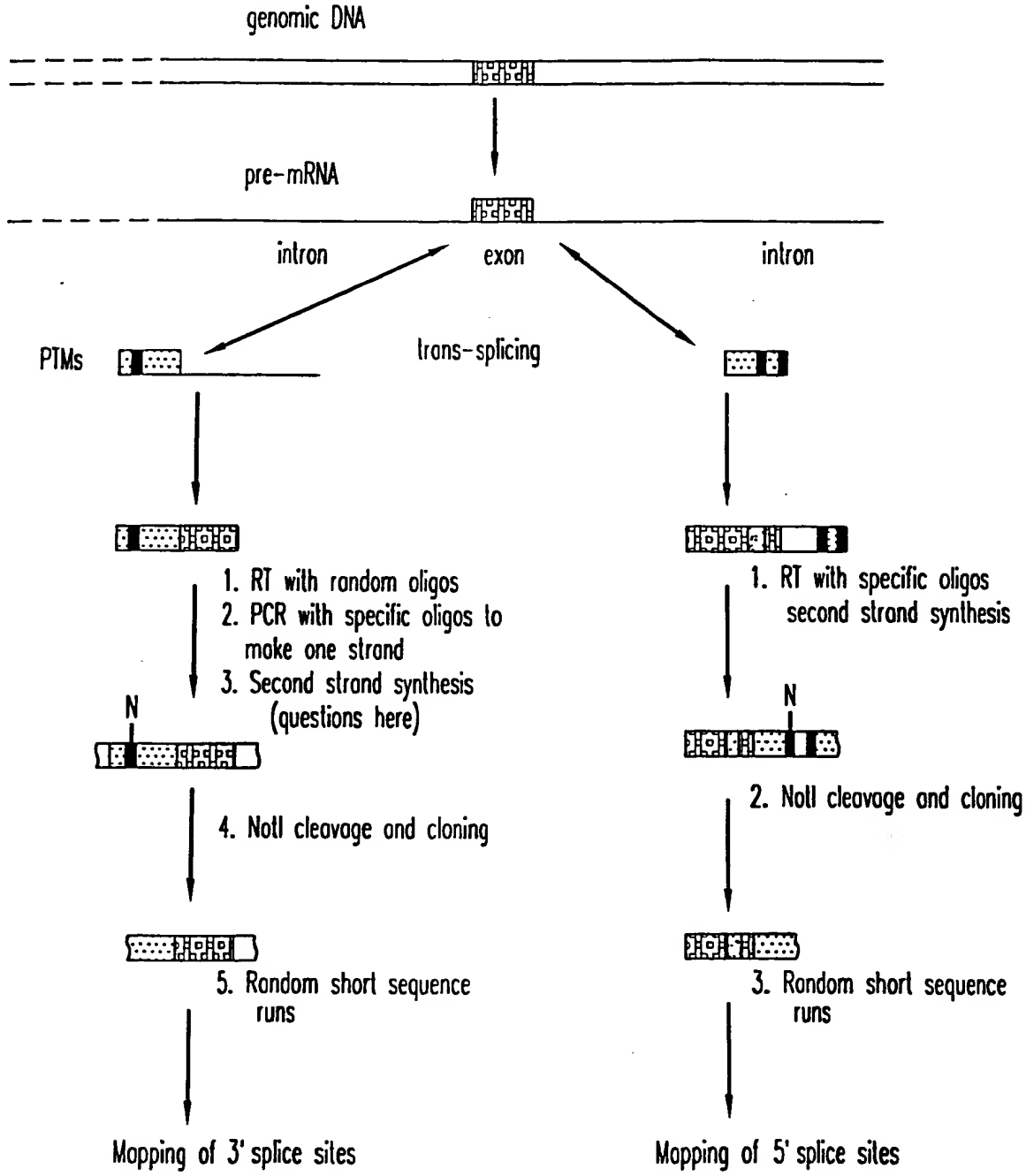
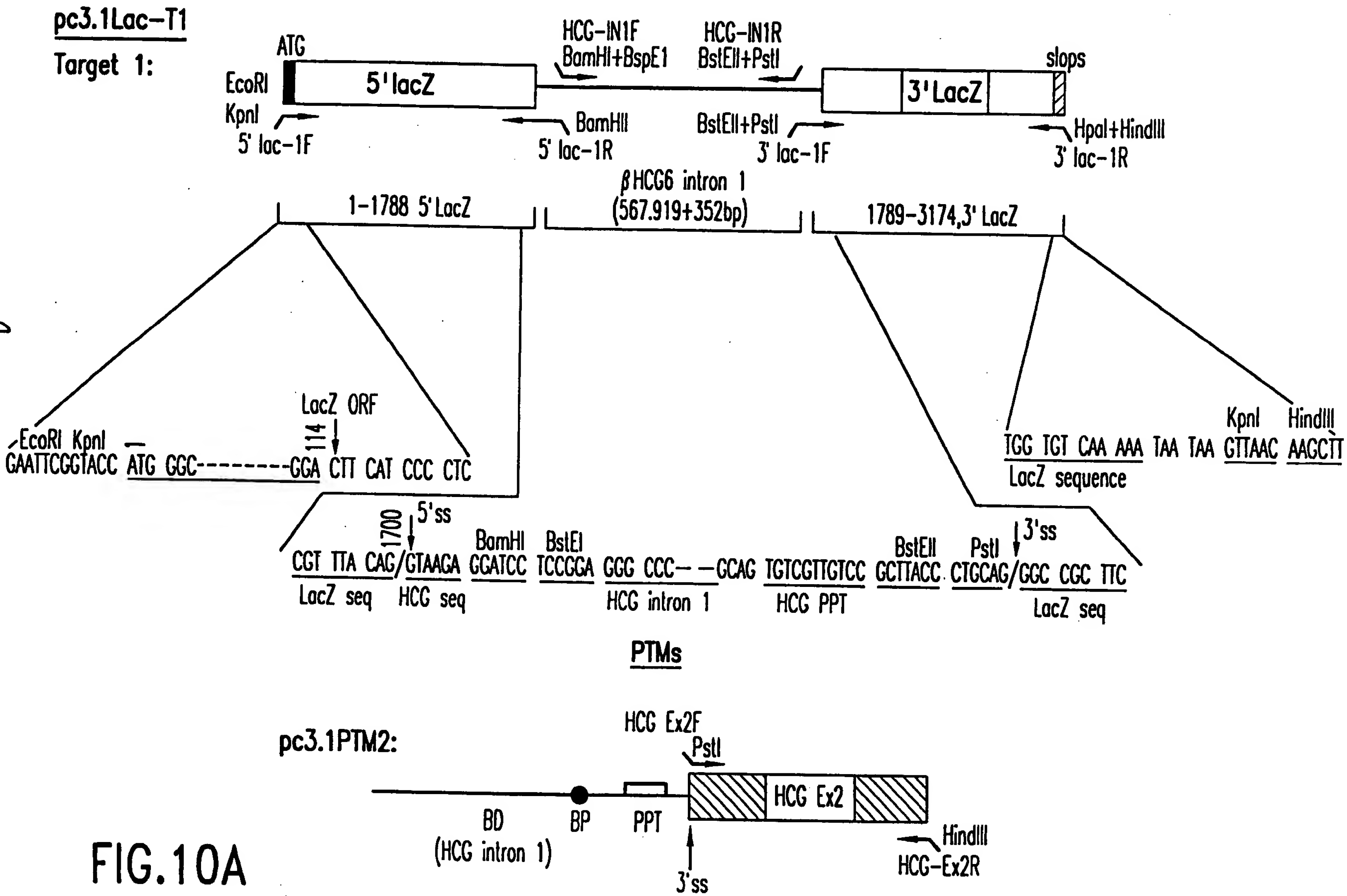


FIG.9



pc3.1Lac-T1
Target 1:
ATG
EcoRI
KpnI
5'lacZ
5' lac-1F
HCG-IN1F
BamHI+BspE1
BamHI
5' lac-1R
HCG-IN1R
BstEII+PstI
BstEII+PstI
3' lac-1F
3'LacZ
slops
HpaI+HindIII
3' lac-1R
1-1788 5'LacZ
βHCG6 intron 1
(567.919+352bp)
1789-3174,3' LacZ
EcoRI KpnI
GAATTCGGTACC ATG GGC--------GGA CTT CAT CCC CTC
LacZ ORF
114
1700
5'ss
CGT TTA CAG/GTAAGA
LacZ seq
HCG seq
BamHI
GGATCC
BstEI
TCCGGA
GGG CCC-- --GCAG
HCG intron 1
TGTCGTTGTCC
HCG PPT
BstEII
GCTTACC
PstI
CTGCAG/GGC CGC TTC
3'ss
LacZ seq
TGG TGT CAA AAA TAA TAA
LacZ sequence
KpnI
GTTAAC
HindIII
AAGCTT
PTMs
pc3.1PTM2:
HCG Ex2F
PstI
HCG Ex2
BD
(HCG intron 1)
BP
PPT
3'ss
HindIII
HCG-Ex2R

FIG.10A

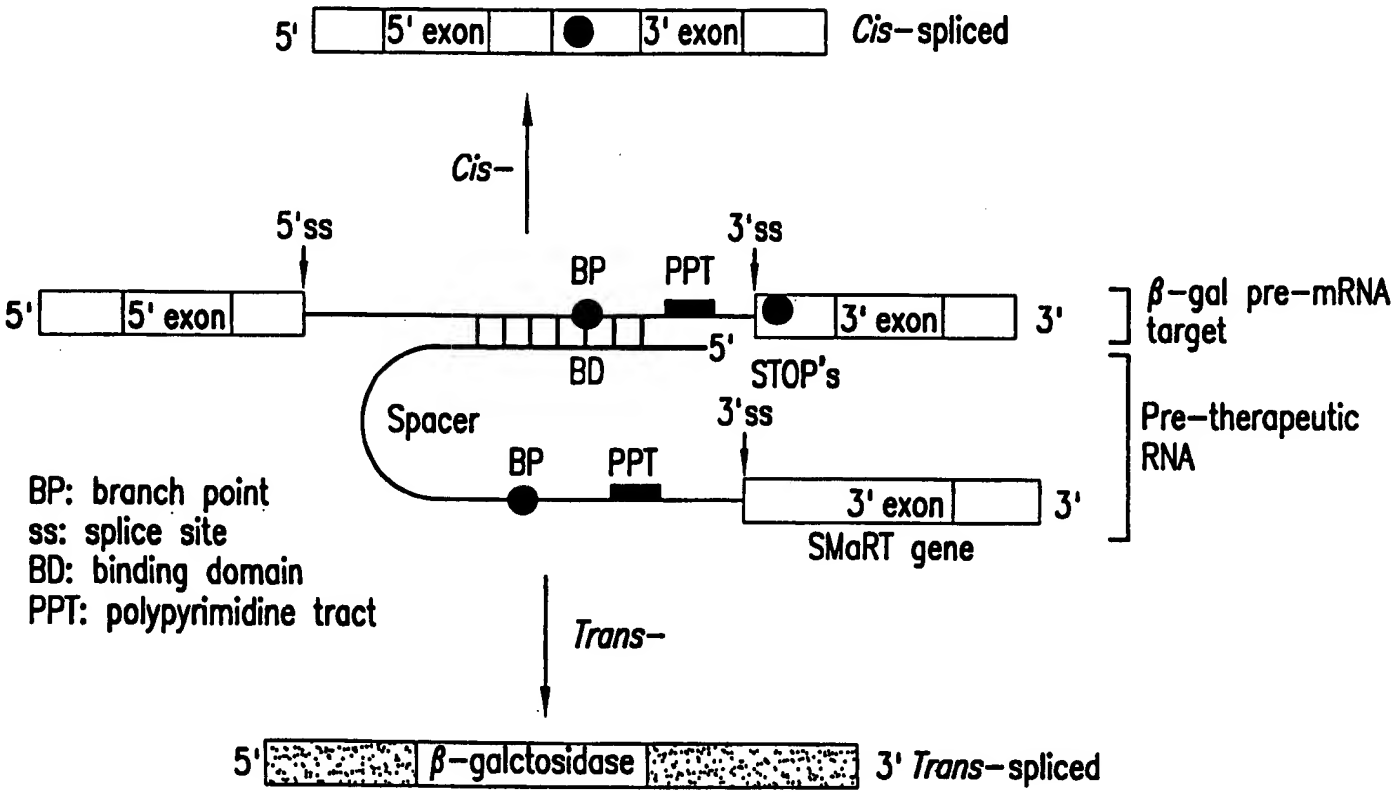


FIG.10B

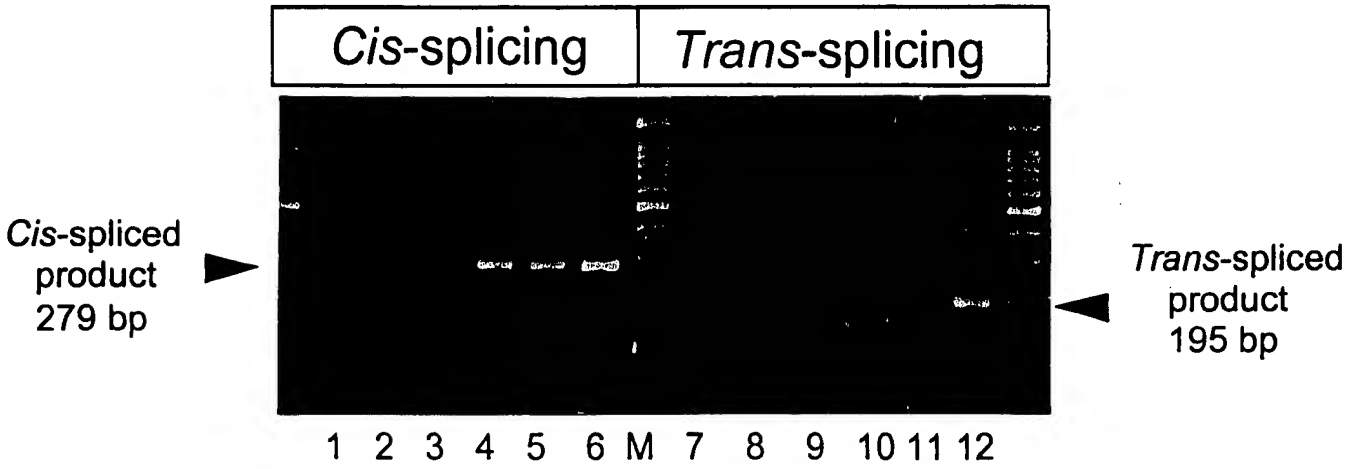


FIG.11A

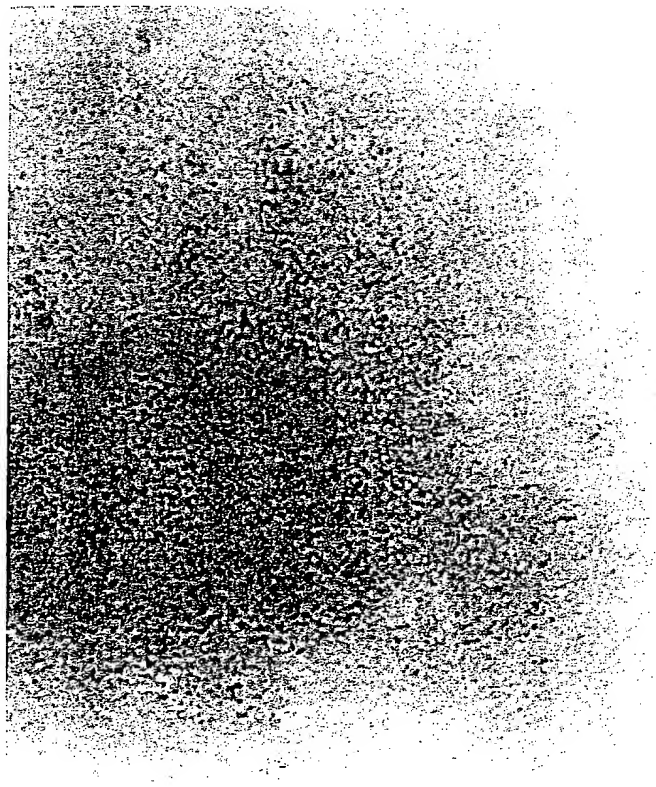


FIG.11B

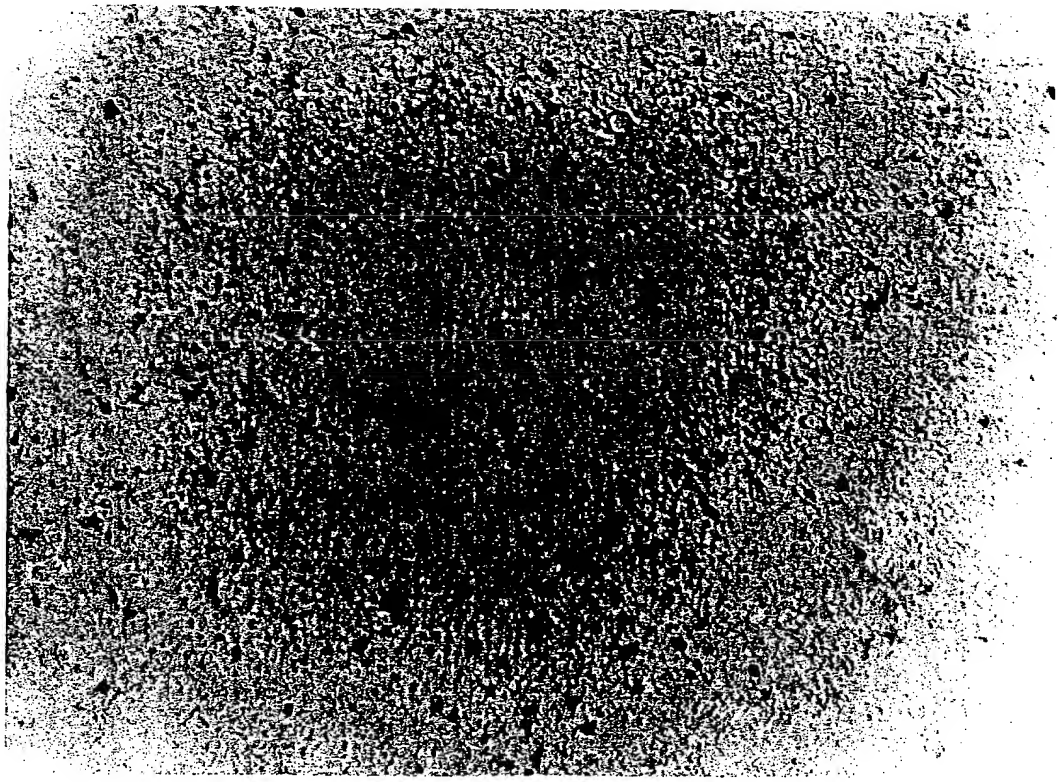


FIG.11C

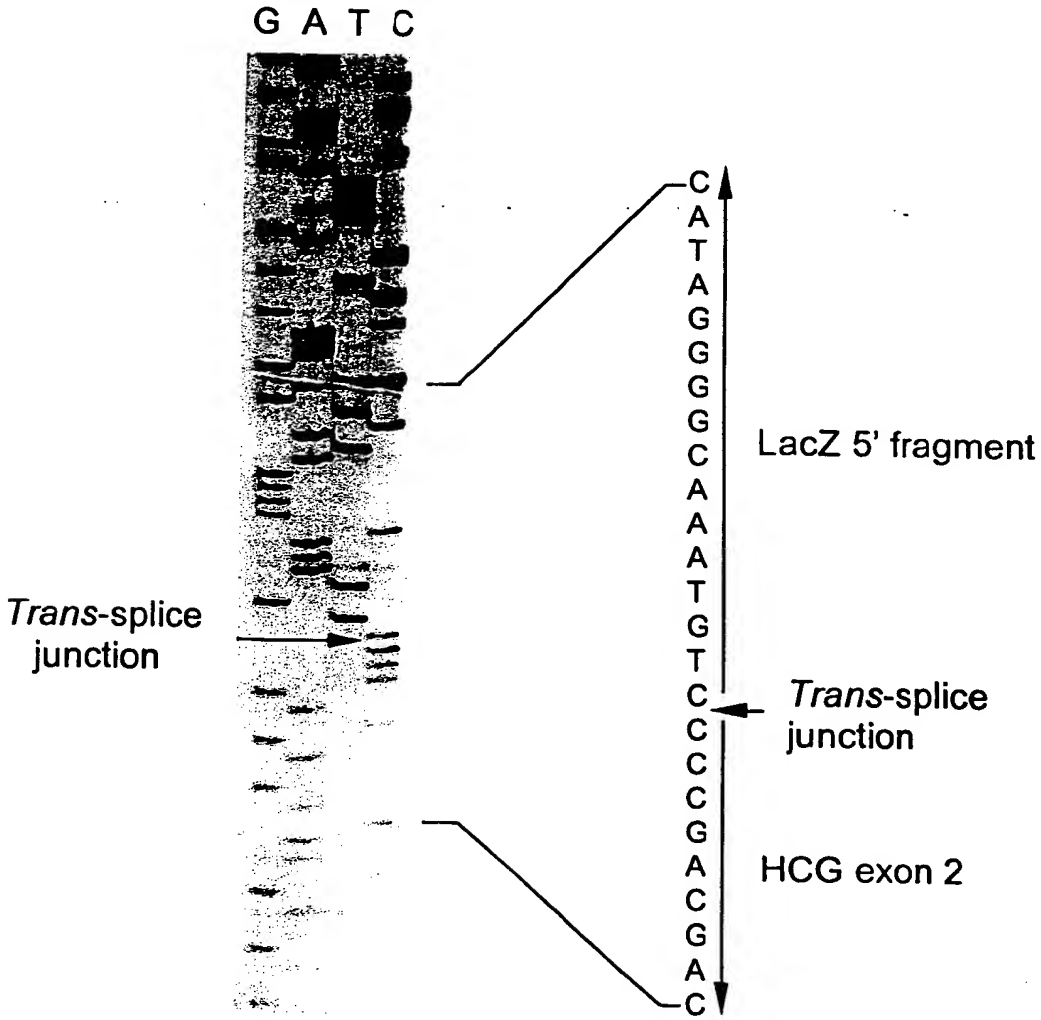


FIG.12A

1. NUCLEOTIDE SEQUENCES OF THE *cis*-SPLICED PRODUCT (285 bp):

BioLac-TR1

GGCTTTCGCTACCTGGAGAGACGCGCCCGCTGATCCTTTGCGAATACGCCCACGCGATGGGTAACAGTCTTG

Splice junction

GCGGTTTCGCTAAATACTGGCAGGCGTTTCGTCAGTATCCCCGTTTACAG/GGCGGCTTCGTCTAATAATG

GGACTGGGTGGATCAGTCGCTGATTAAATATGATGAAAACGGCAACCCGTGGTCGGCTTACGGCGGTGATTT

Lac-TR2

TGGCGATACGCCGAACGATCGCCAGTTCTGTATGAACGGTCTGGTCTTTGCCGACCGCACGCCGCATCCAG

2. NUCLEOTIDE SEQUENCES OF THE *trans*-SPLICED PRODUCT (195 bp)

BioLac-TR1

GGCTTTCGCTACCTGGAGAGACGCGCCCGCTGATCCTTTGCGAATACGCCCACGCGATGGGTAACAGTCTTGG

Splice junction

CGGTTTCGCTAAATACTGGCAGGCGTTTCGTCAGTATCCCCGTTTACAG/GGGCTGCTGCTGTTGCTGCTGCT

HCGR2

GAGCATGGGCGGGACATGGGCATCCAAGGAGCCACTTCGGCCACGGTGCCG

FIG.12B

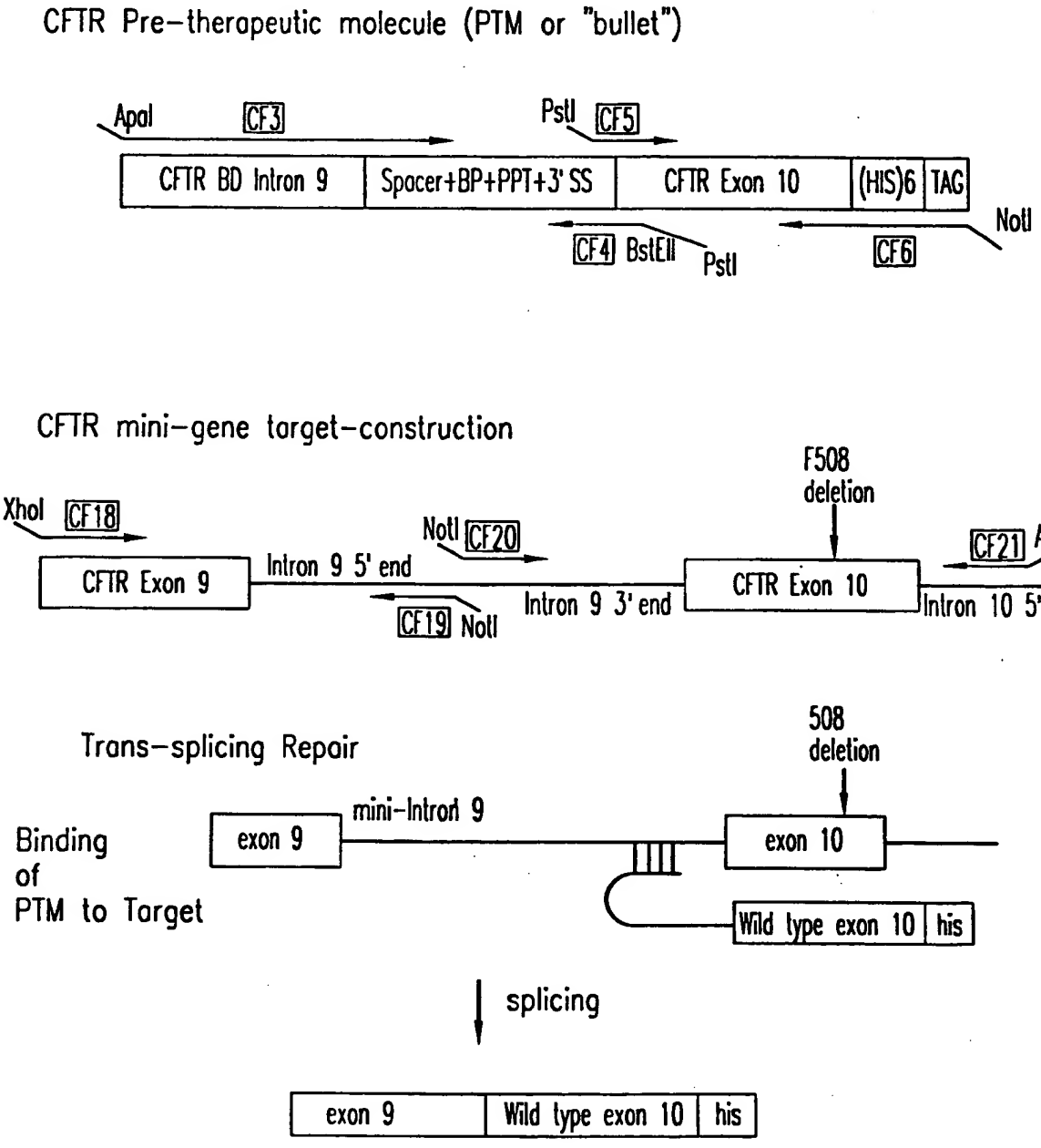

CFTR Pre-therapeutic molecule (PTM or "bullet")
ApaI
CF3
PstI
CF5
CFTR BD Intron 9
Spacer+BP+PPT+3' SS
CFTR Exon 10
(HIS)6
TAG
NotI
CF4
BstEII
PstI
CF6
CFTR mini-gene target-construction
F508 deletion
XhoI
CF18
NotI
CF20
CF21
AflII
CFTR Exon 9
Intron 9 5' end
Intron 9 3' end
CFTR Exon 10
Intron 10 5' end
CF19
NotI
Trans-splicing Repair
508 deletion
Binding of PTM to Target
exon 9
mini-Intron 9
exon 10
Wild type exon 10
his
splicing
exon 9
Wild type exon 10
his

FIG.13

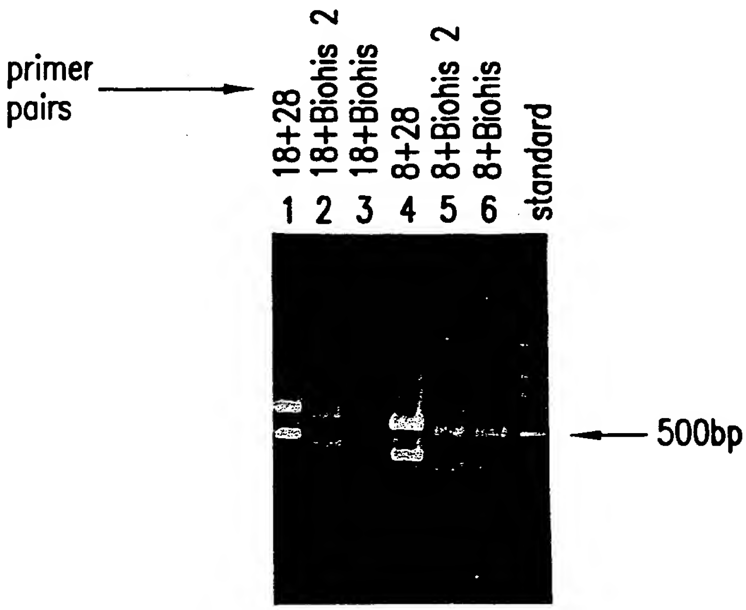


FIG.14

DNA sequence 500 b.p. GCTAGCGTTTAA ... TGCCACTCCCAC linear

Positions of Restriction Endonucleases sites (unique sites underlined)

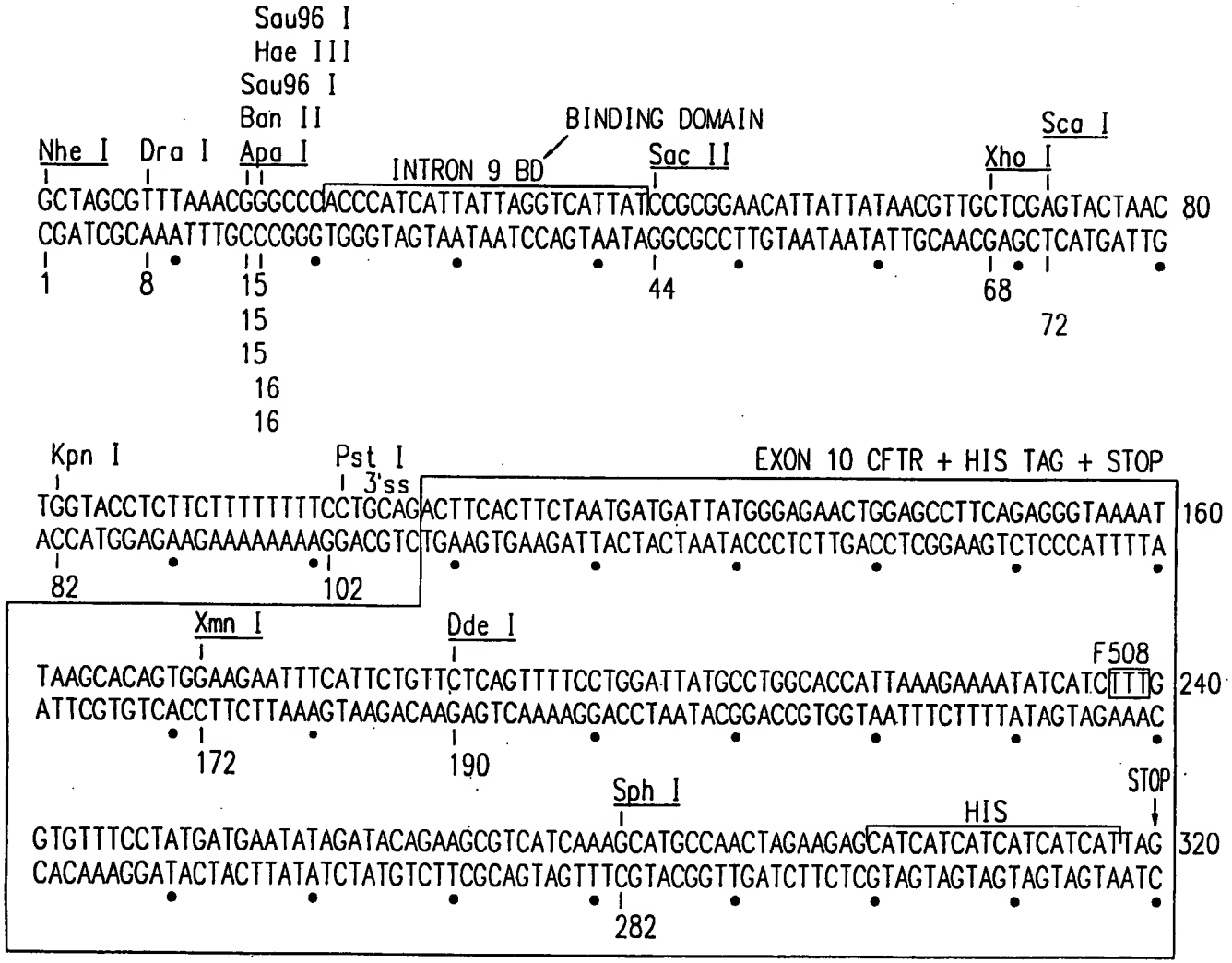
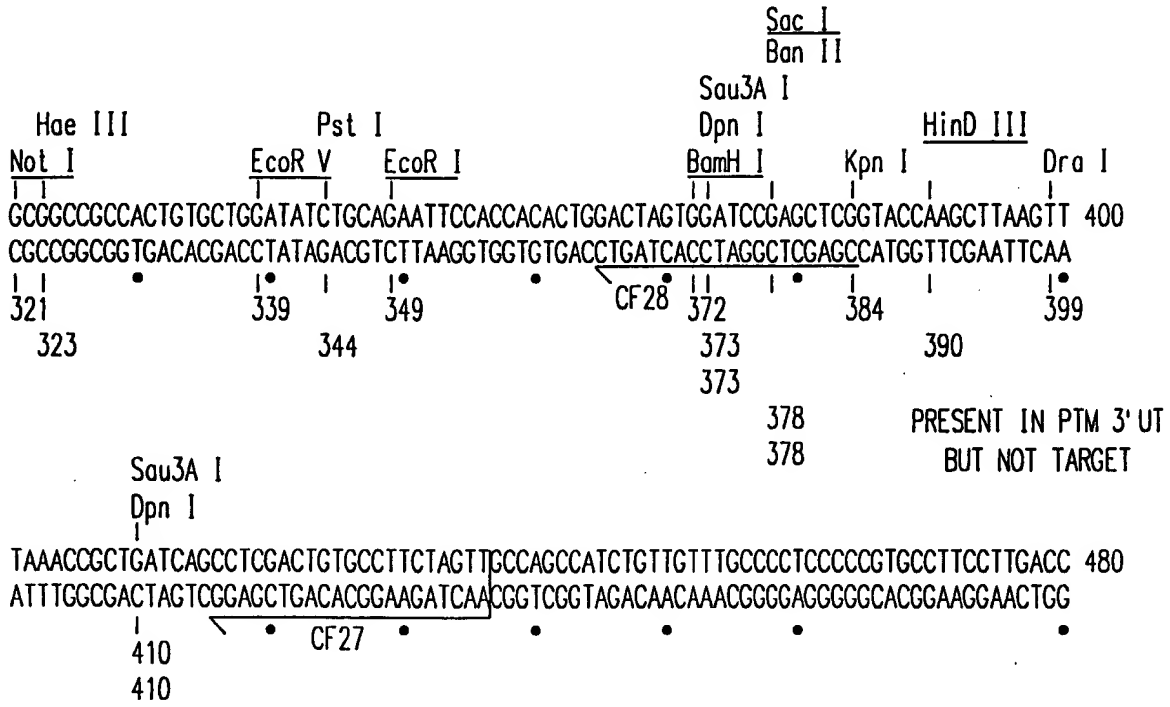


FIG.15A



Restriction Endonucleases site usage

Acc I	–	EcoR I	1	Nde I	–	Sau96 I	2
Apa I	1	EcoR V	1	Nhe I	1	Sca I	1
ApaL I	–	Hae II	–	Not I	1	Sma I	–
Avr II	–	Hae III	2	PflM I	–	Sph I	1
BamH I	1	HinC II	–	Pst I	2	Spl I	–
Ban II	2	HinD III	1	Pvu I	–	Ssp I	–
Bbe I	–	Hinf I	–	Pvu II	–	Stu I	–

FIG.15B

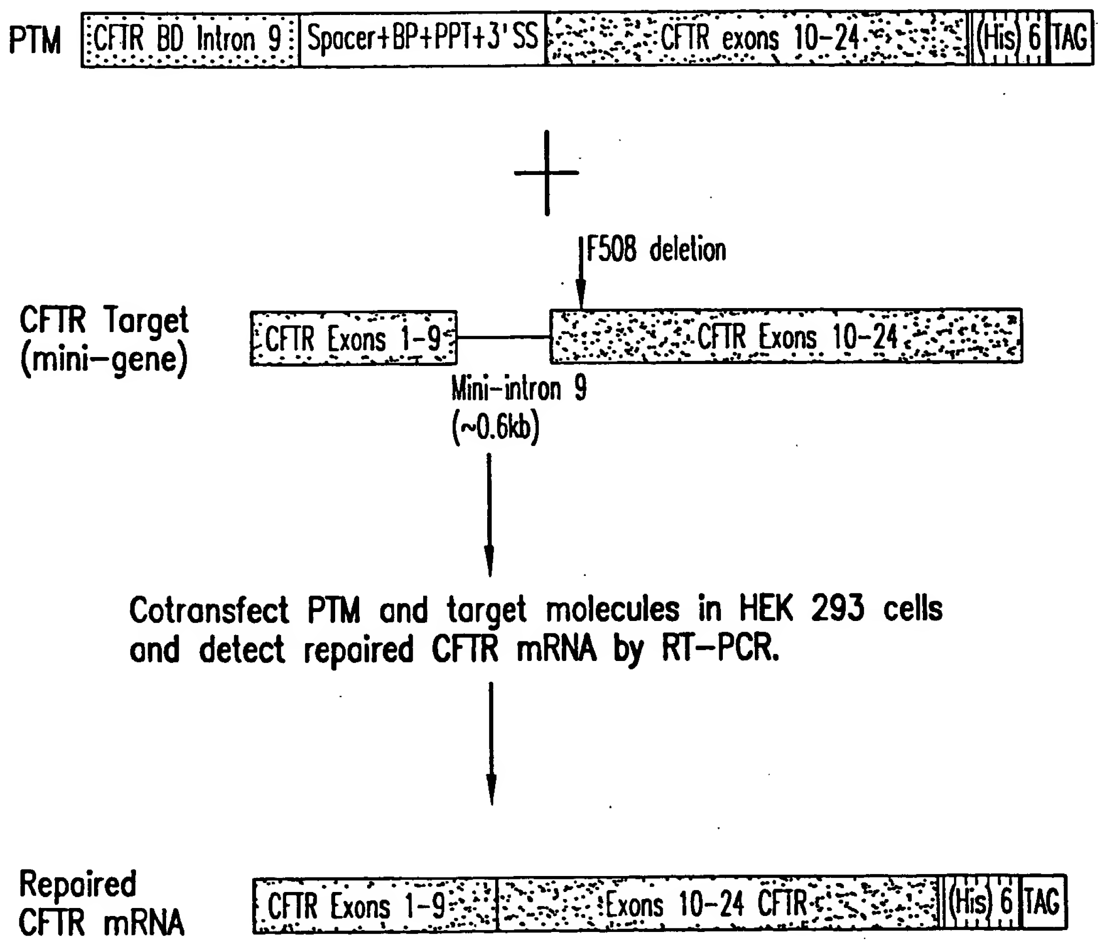


FIG.16

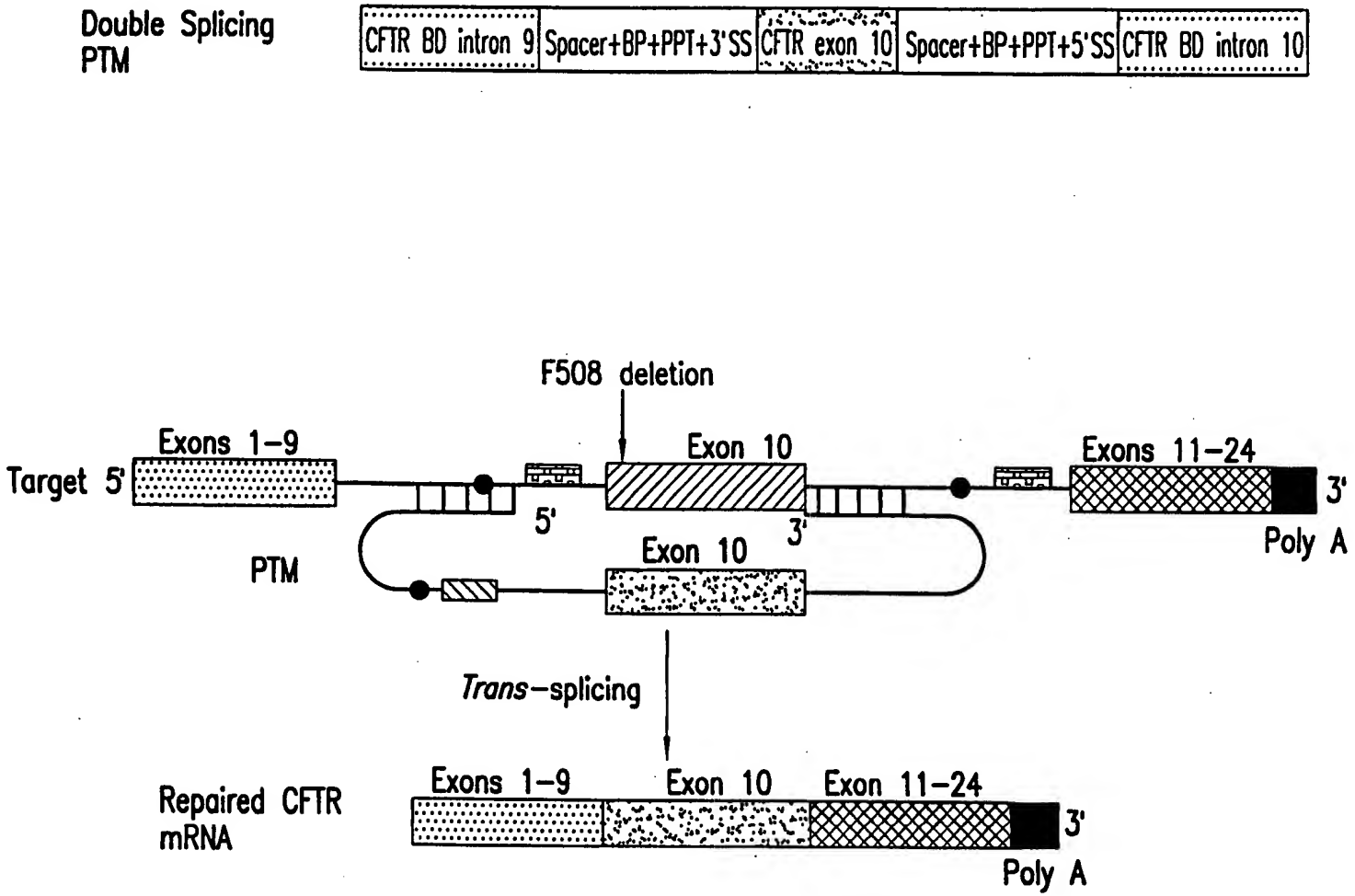
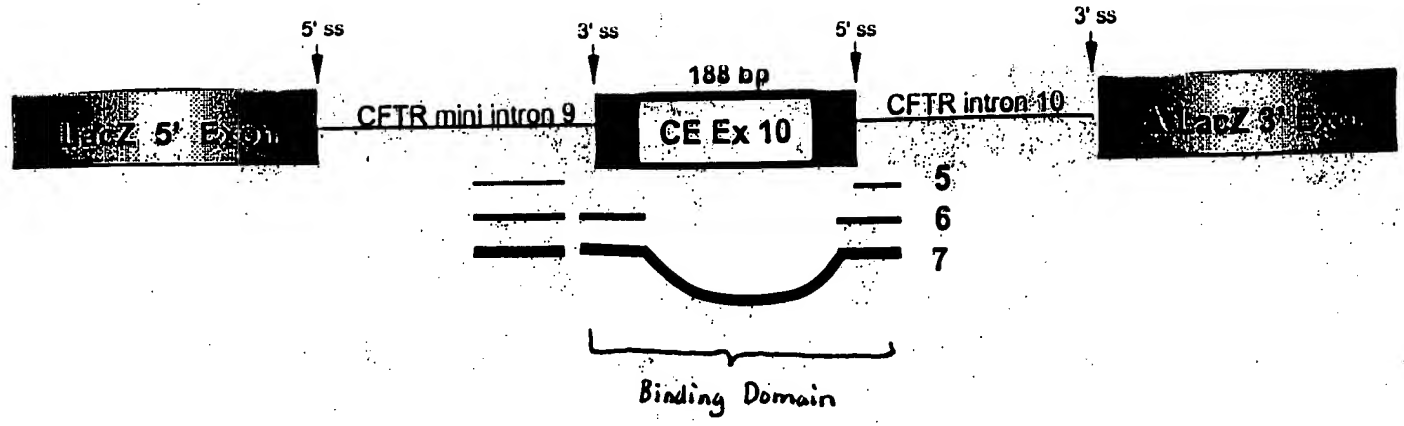

Double Splicing PTM
CFTR BD intron 9
Spacer+BP+PPT+3'SS
CFTR exon 10
Spacer+BP+PPT+5'SS
CFTR BD intron 10
F508 deletion
Exons 1–9
Target 5'
Exon 10
Exons 11–24
3'
5'
3'
Poly A
PTM
Exon 10
Trans–splicing
Repaired CFTR mRNA
Exons 1–9
Exon 10
Exon 11–24
3'
Poly A

FIG.17

Double *Trans*-splicing Specific Target



Target
DSCFT1-6

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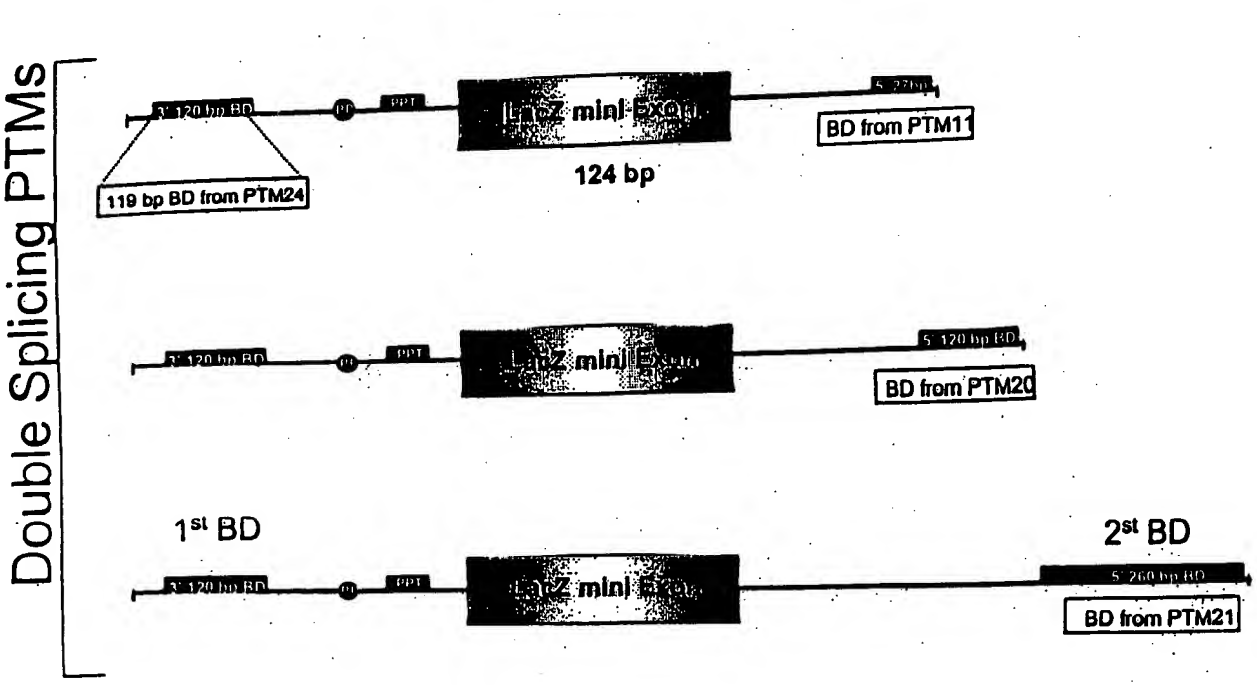
Figure 18

Double *Trans*-splicing PTMs



DSPTM-5

PTM with 27 bp BD & masks 5' single splice site

DSPTM-6

PTM with 120 bp BD & masks both 5' & 3' splice sites

DSPTM-7

PTM with 260 bp BD masking both the ss & the entire CFTR Ex10

Figure 19

Double *Trans*-splicing β-Gal Model

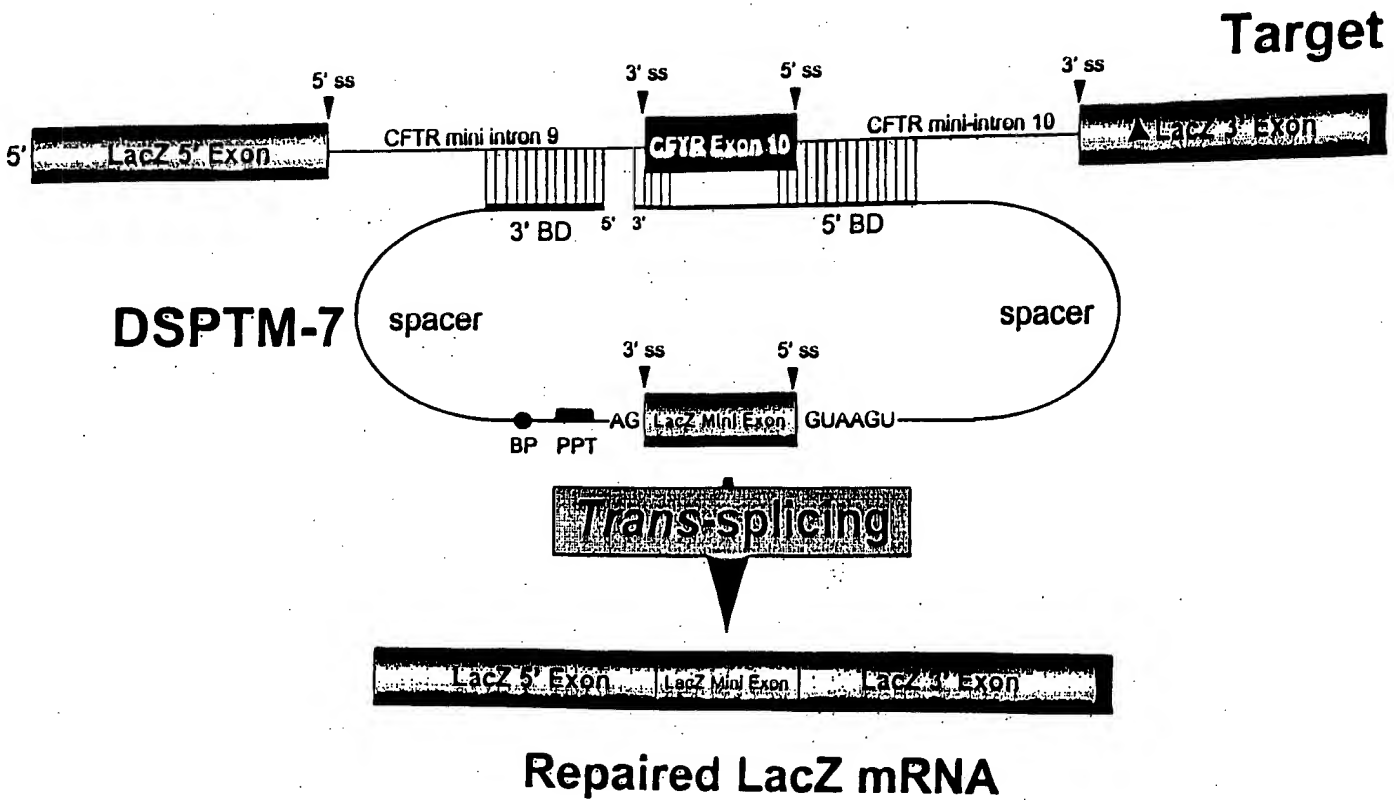
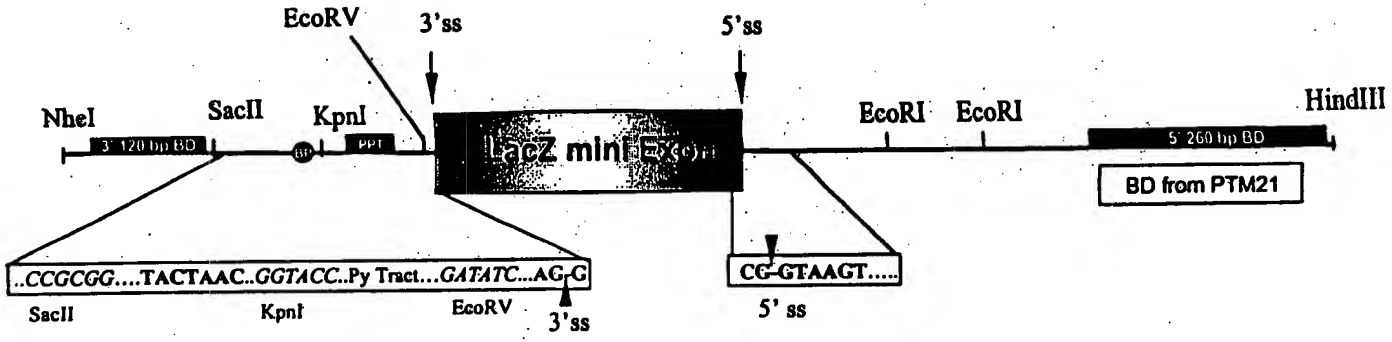


Figure 20

Important Structural Elements of DSPTM-7: (Double splicing PTM with all the necessary splice elements i.e. has both 3' and 5' functional splice sites and the binding domains)



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(1) 3' BD (120 BP) : GATTCACTTGCTCCAATTATCATCCTAAGCAGAAGTGTATATTCTTATTTGTAAAGATTCTATTAACTCATTTGATTC
AAAATATTTAAAATACTTCCTGTTTCATACTCTGCTATGCAC

(2) Spacer sequences (24 bp): AACATTATTATAACGTTGCTCGAA

(3) Branch point, pyrimidine tract and acceptor splice site: TACTAAC T *GGTACC* TCTTCTTTTTTTTTT *GATATC* CTGCAG GGC GCG

BP Kpn I PPT EcoRV 3' ss LacZ mini exon

(4) 5' donor site and 2nd spacer sequence: GTA AGG *GTAAGT* GTTATCACCGATATGTGTCTAACCTGATTCGGGCCTTCGATACG

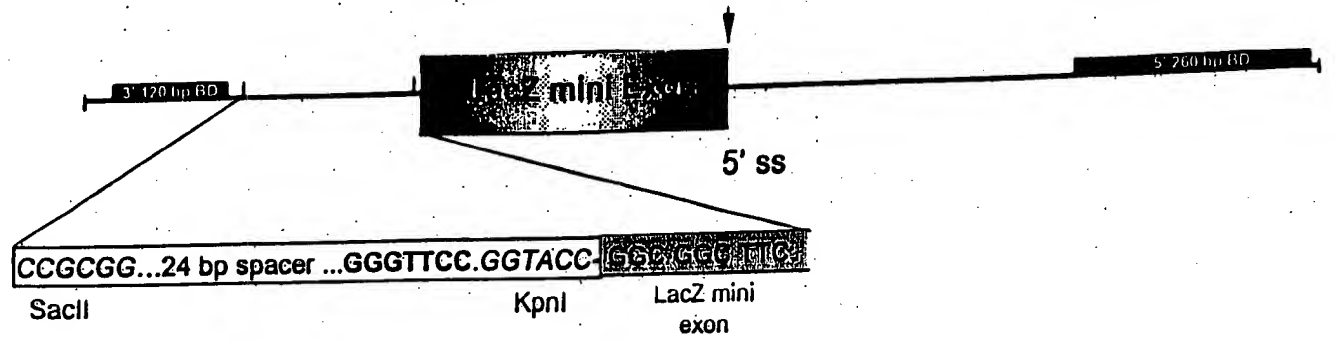
CTAAGATCCACCGG

LacZ mini exon 5' ss

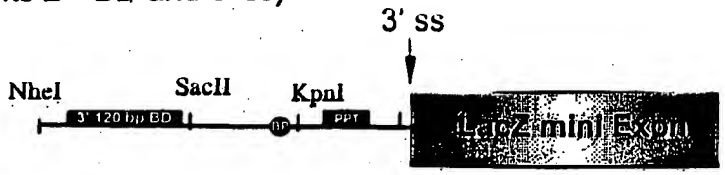
(5) 5' BD (260 BP) : TCAAAAAGTTTTCACATAATTTCTTACCTCTTCTT*GAATTC*ATGCTTTGATGACGCTTCTGTATCTATATTCATCATTGGAA
ACACCAATGATTTTTCTTTAATGGTGCCTGGCATAATCCTGGAAAACTGATAACACAATGAAATTCTTCCACTGTGCTTAA
AAAAACCCTCT*GAATTC*TCCATTTCTCCCATAATCATCATTACAACTGAACTCTGGAAATAAAACCCATCATTATTAACTCA
TTATCAAATCACGC

Figure 21

DSPTM8 :(▲ 3' ss: 3' splice elements i.e. BP, PPT & AG dinucleotide has been deleted and replaced with random sequences, but still has the functional 5' splice site)



PTM29 (lacks 2nd BD and 5' ss)



PTM30 (lacks 1st BD and 3' ss)

Mutants

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Figure 22

Accuracy of Double *Trans*-splicing Reaction

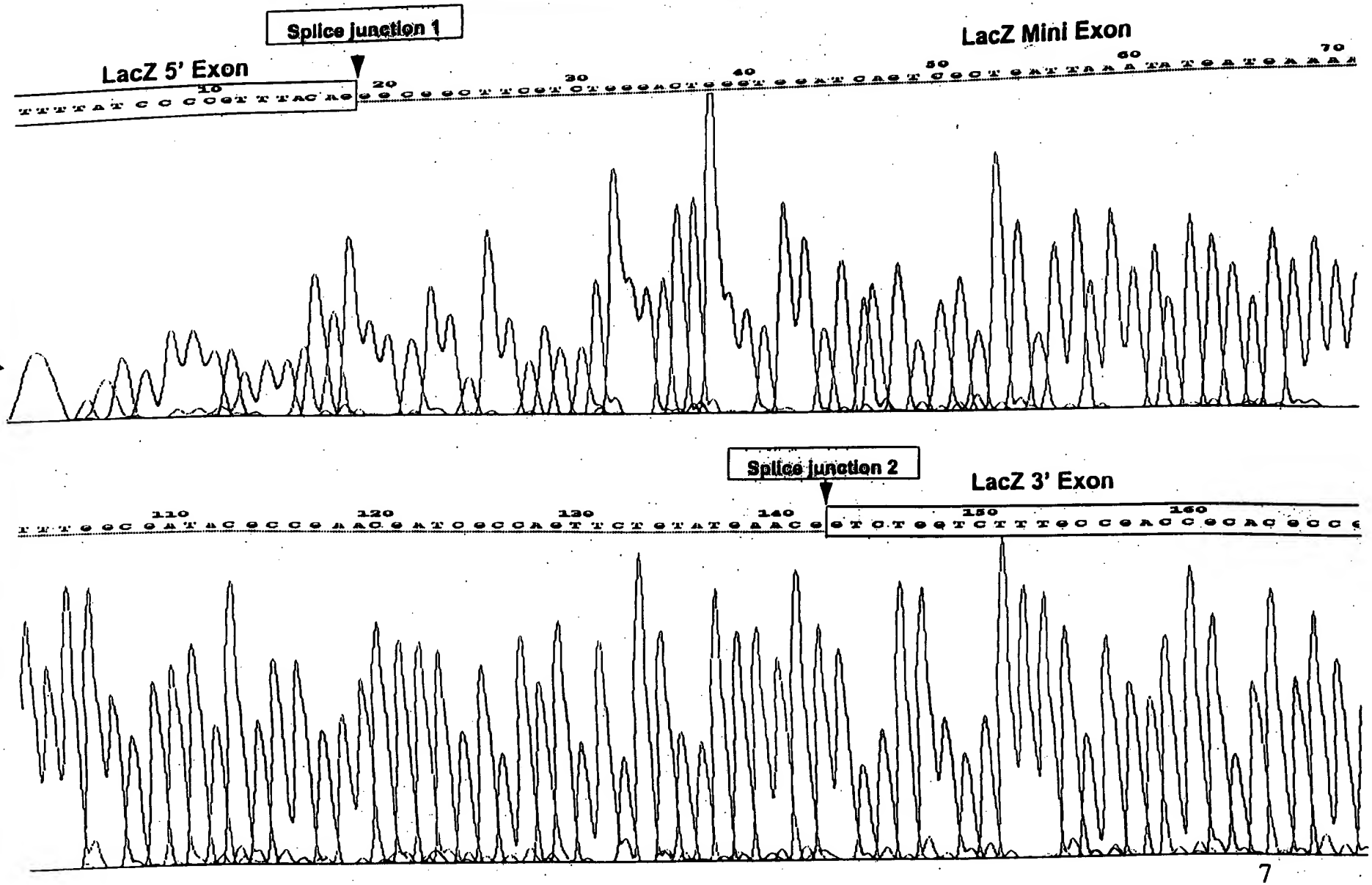
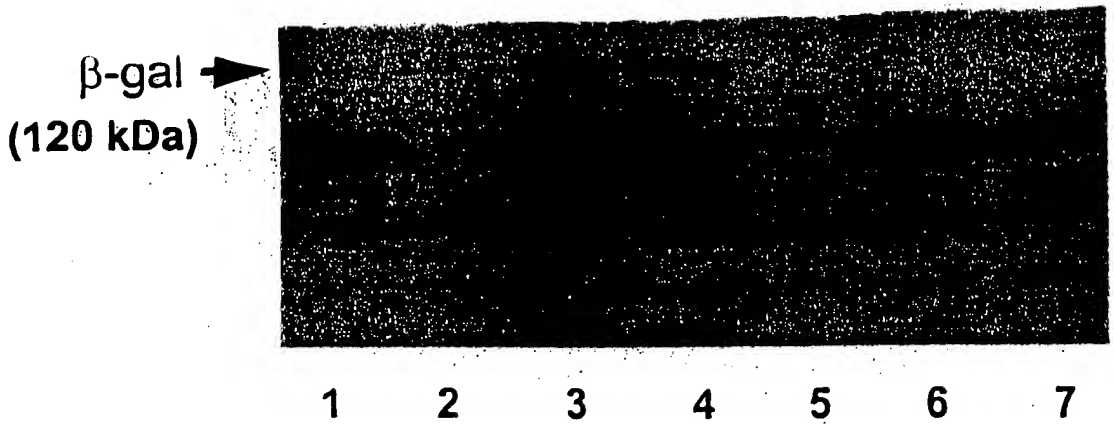


Figure 23

Double *Trans*-splicing Produces Full-length Protein



Lane 1: DSCFT1.6 Target alone	25 µg
Lane 2: DSPTM7	25 µg
Lane 3 Target + PTM #6	25 µg
Lane 4: Target + PTM #9	25 µg
Lane 5: Delta 3' splice mutant alone	25 µg
Lane 6: Target + Delta 3' ss	25 µg
Lane 7: Target+PTM29+30 (mutants)	25 µg

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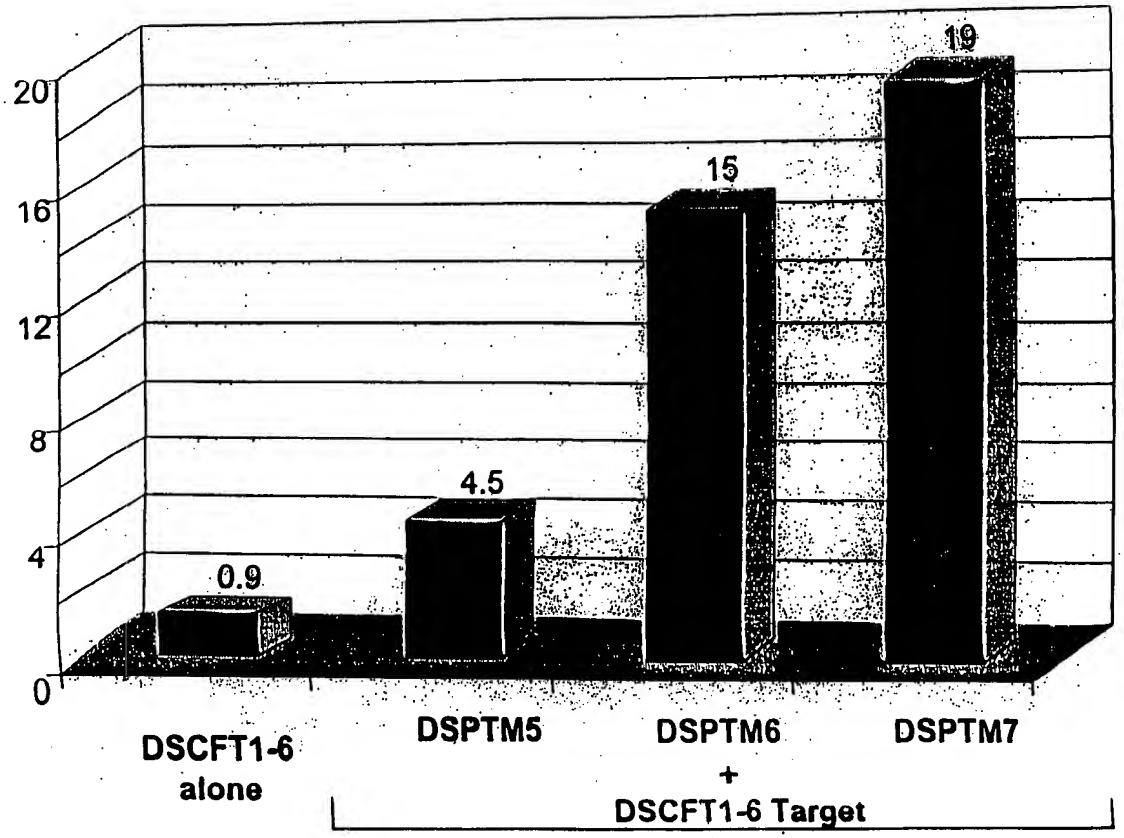
Figure 24

Restoration of β-Gal Function by Double *Trans*-splicing



Beta-gal Activity above the Background level

DSPTM5: 5 fold
DSPTM6: 17 fold
DSPTM7: 21 fold

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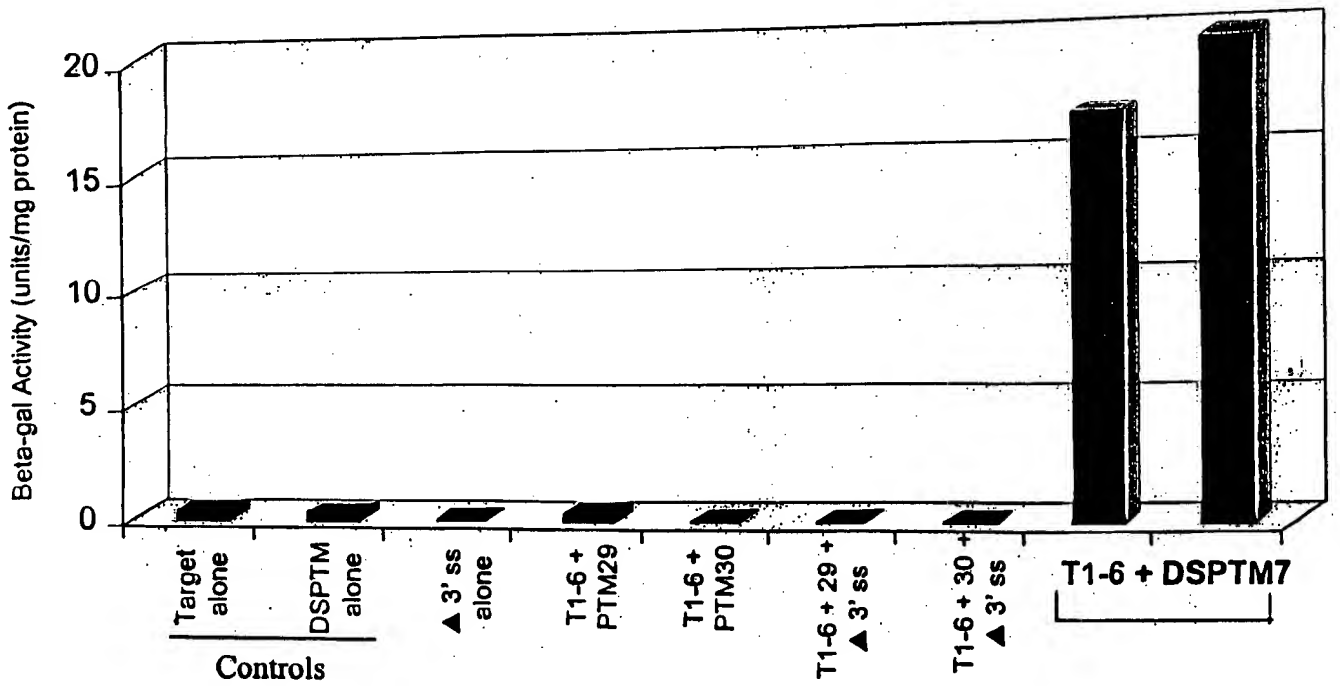
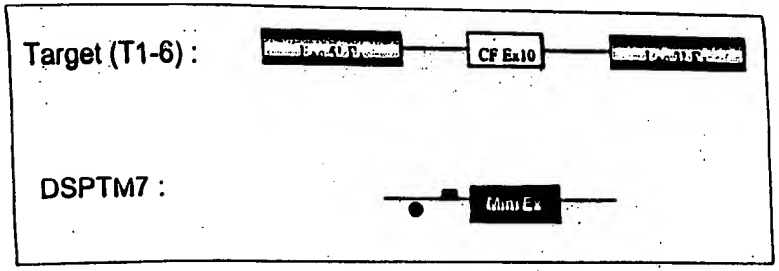
Figure 25

Restoration of β-gal activity is due to double RNA *trans*-splicing events





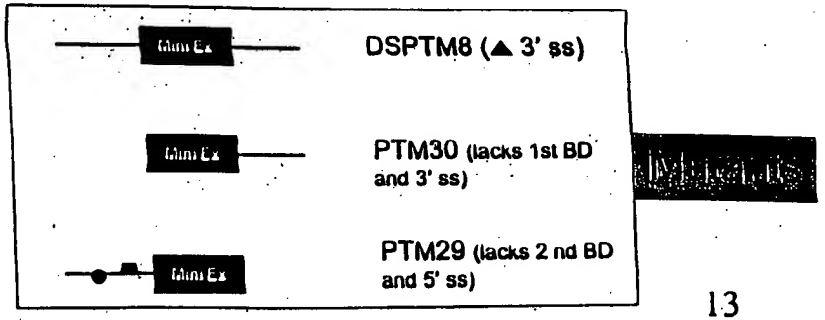
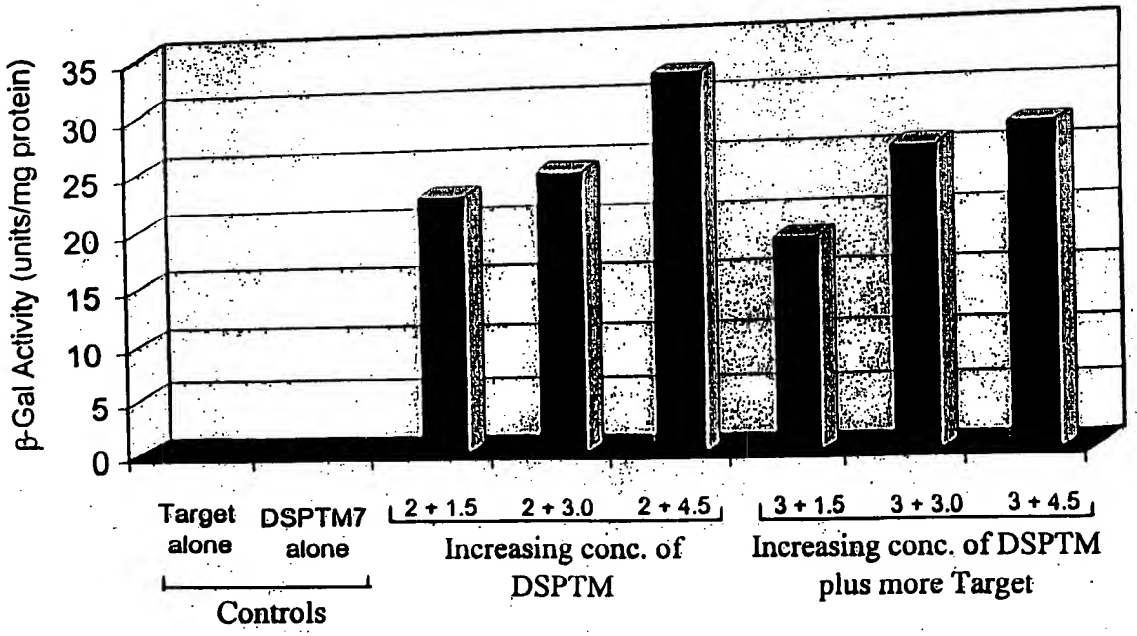


Figure 26

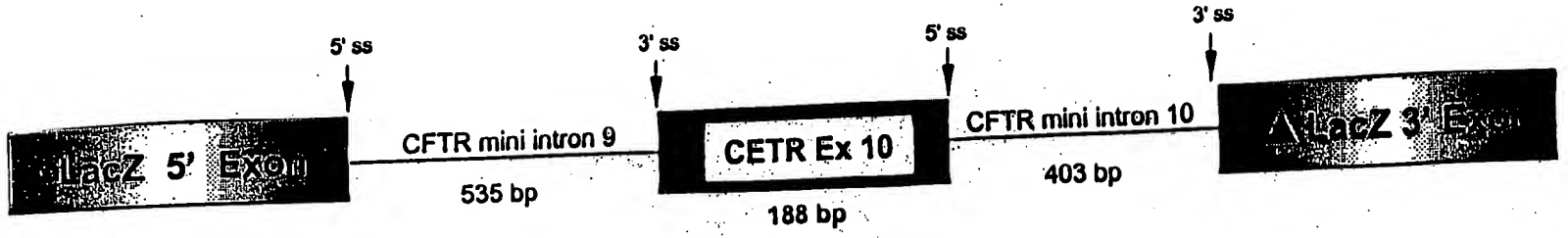
Double *Trans*-splicing: Titration of Target & PTM



The current level of beta-gal activity due to double *trans*-splicing is ~ 1-1.5% of the best single splice model (3' exon replacement)

Figure 27

DSCFT1-6 (Specific Target):



DSHCGT1 (Non-specific Target):

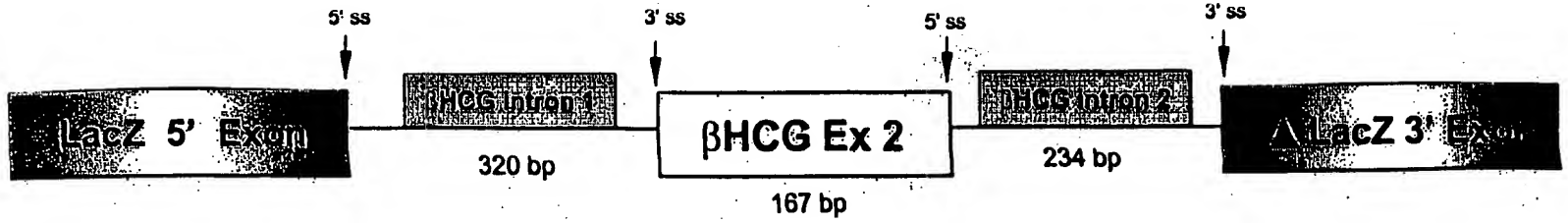


Figure 28

Specificity of double *trans*-splicing Reaction

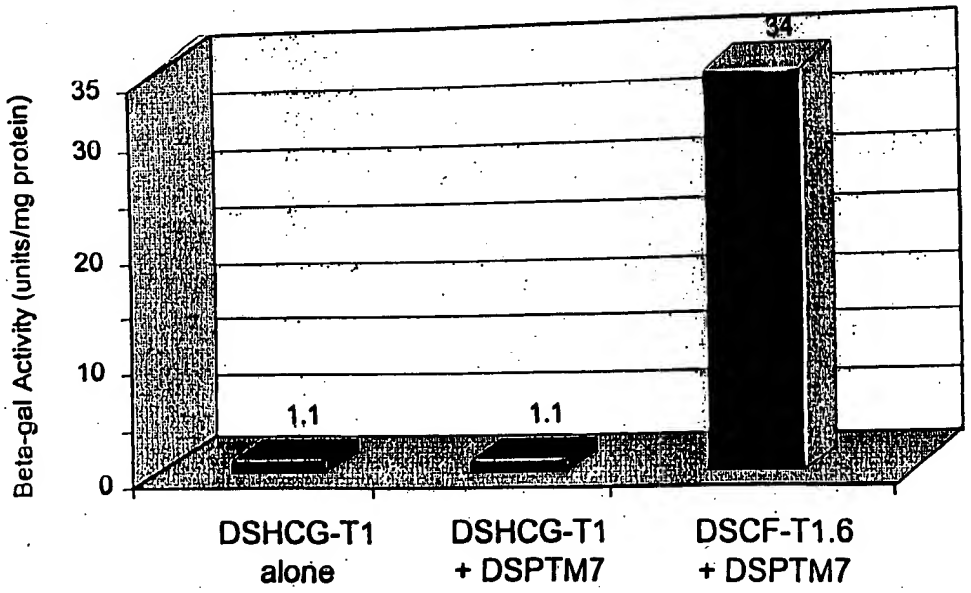


Figure 29

Replacement of a Single Internal Exon

Schematic diagram of a PTM binding to a CFTR ΔF508 target

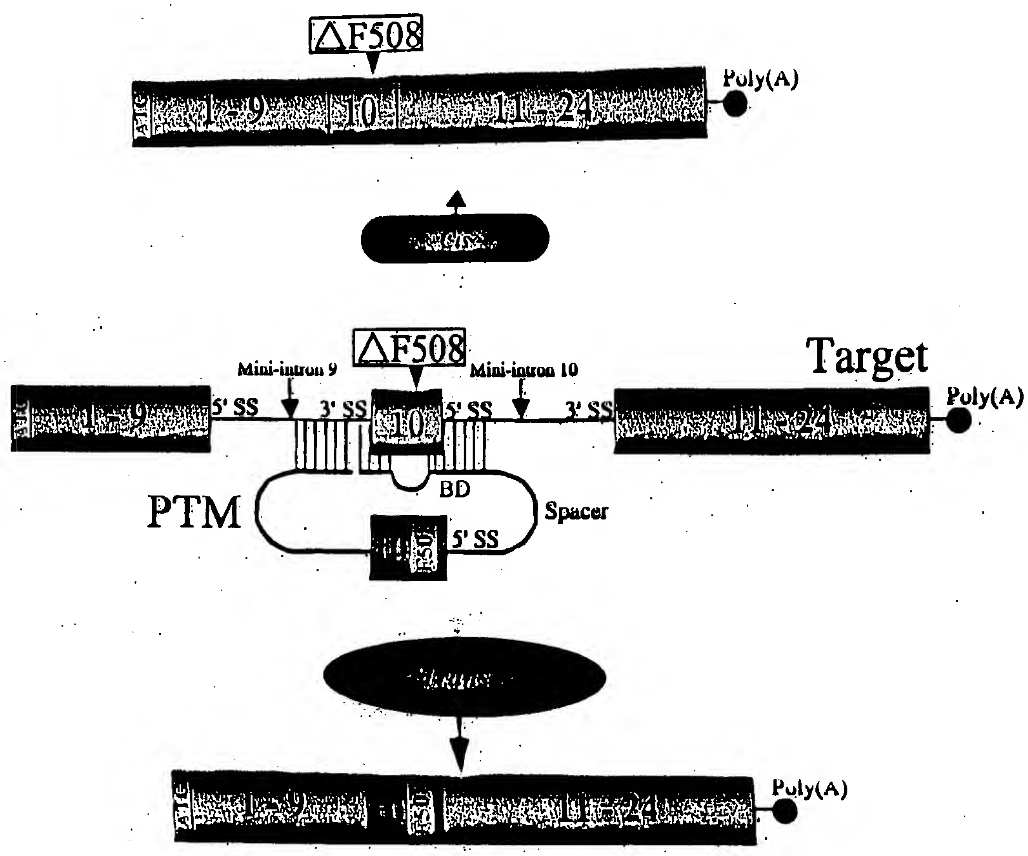


Figure 30

INTRONN

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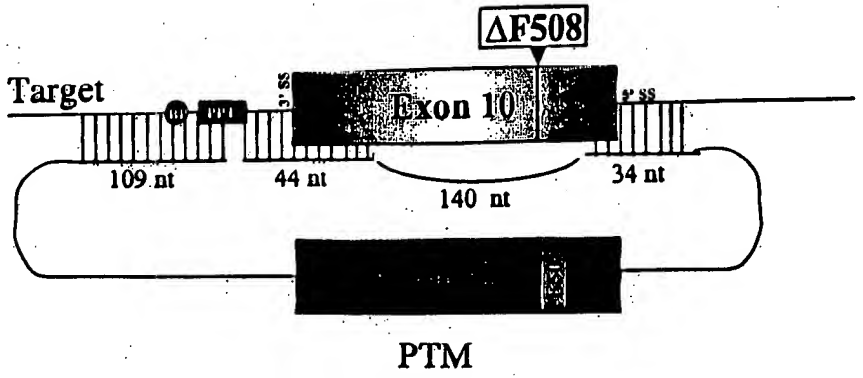
PTM with a long binding domain masking two splice sites and part of exon 10 in a mini-gene target.



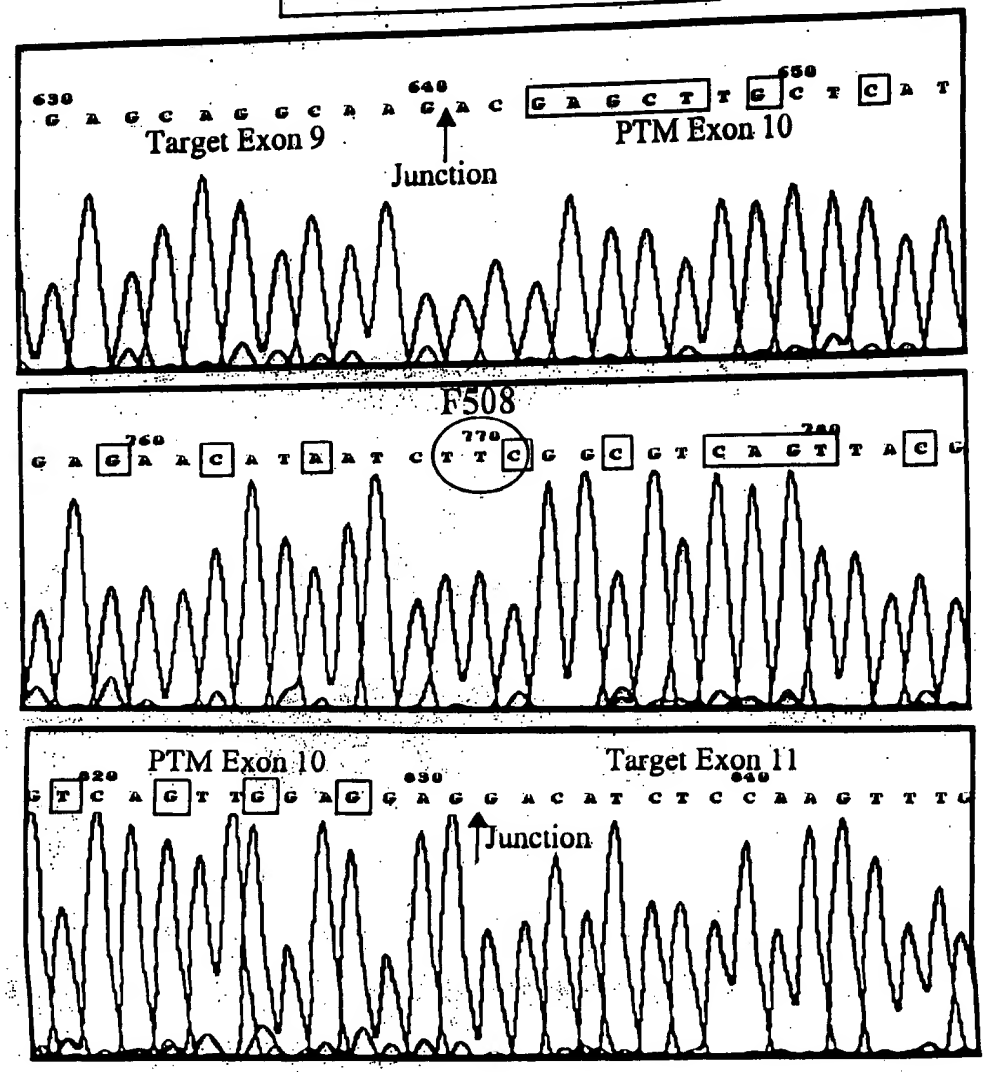
ACGAGCTTGCTCATGATGATCATGGGCGAGTTAGAACCAAGTGAAGGCAAGATCAAACATTCCG
GCCGCATCAGCTTTTGCAGCCAATTCAGTTGGATCATGCCCGGTACCATCAAGGAGAACATAAT
CTTCGGCGTCAGTTACGACGAGTACCGCTATCGCTCGGTGATTAAGGCCTGTCAGTTGGAGGAG

MCU in exon 10 of PTM

88 of 192 (46%) bases in PTM exon 10 are not complementary to its binding domain (bold and underlined).

Figure 31

Sequence of a double *trans*-spliced product

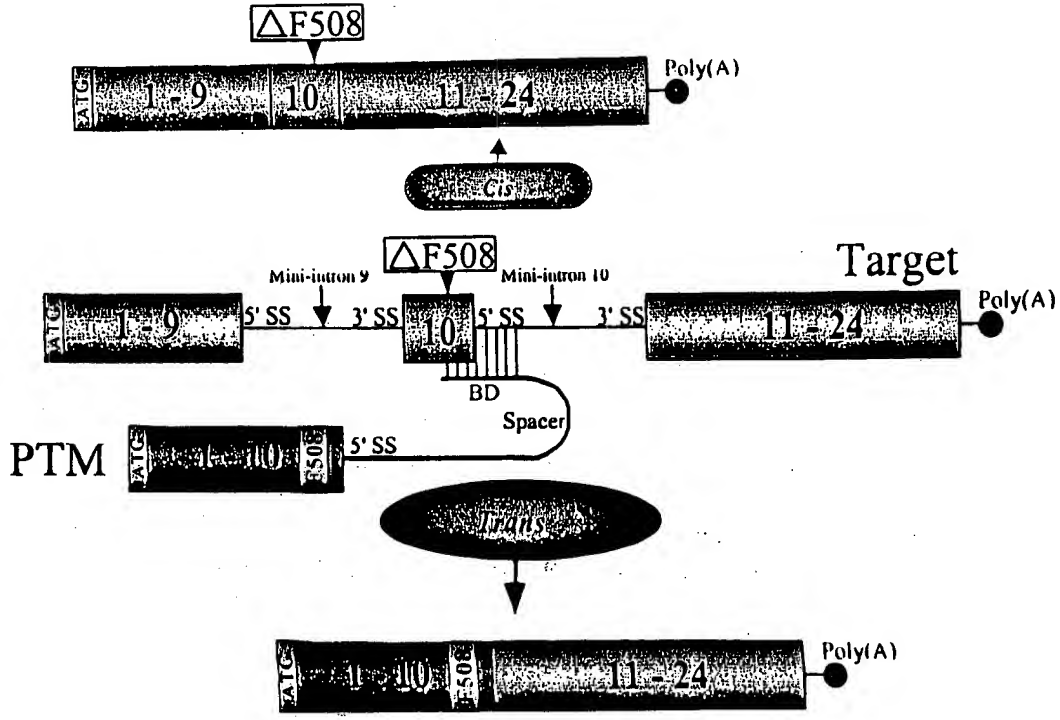


☐ = MCU in PTM exon 10

Figure 32

CFTR Repair: 5' Exon Replacement

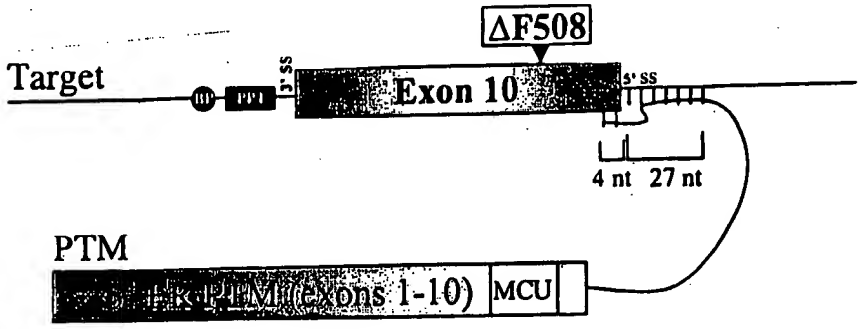
Schematic diagram of a PTM binding to the splice site of intron 10 of a mini-gene target



Repaired full length CFTR mRNA

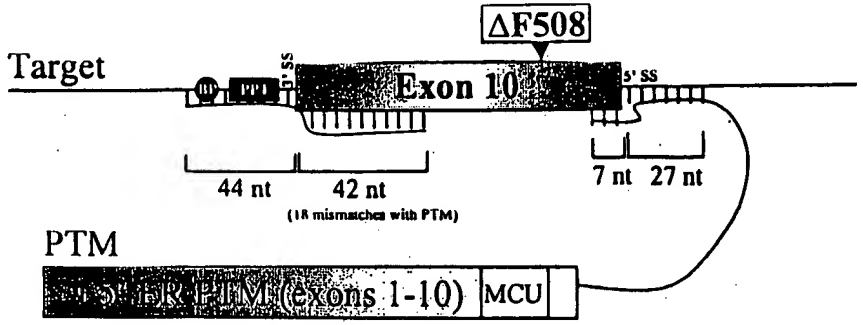
Figure 33

A



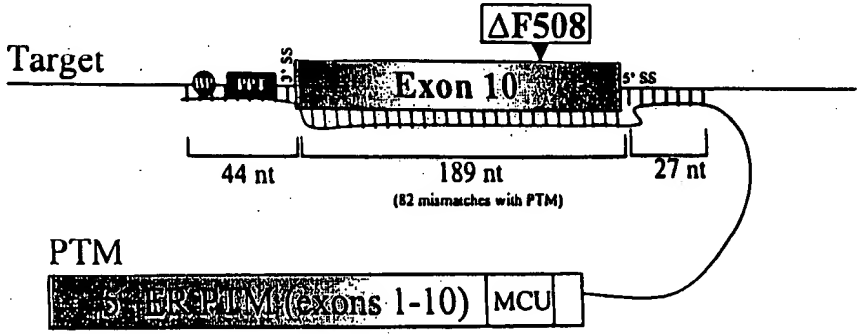
PTM with a short binding domain masking a single splice site in a mini-gene target.

B



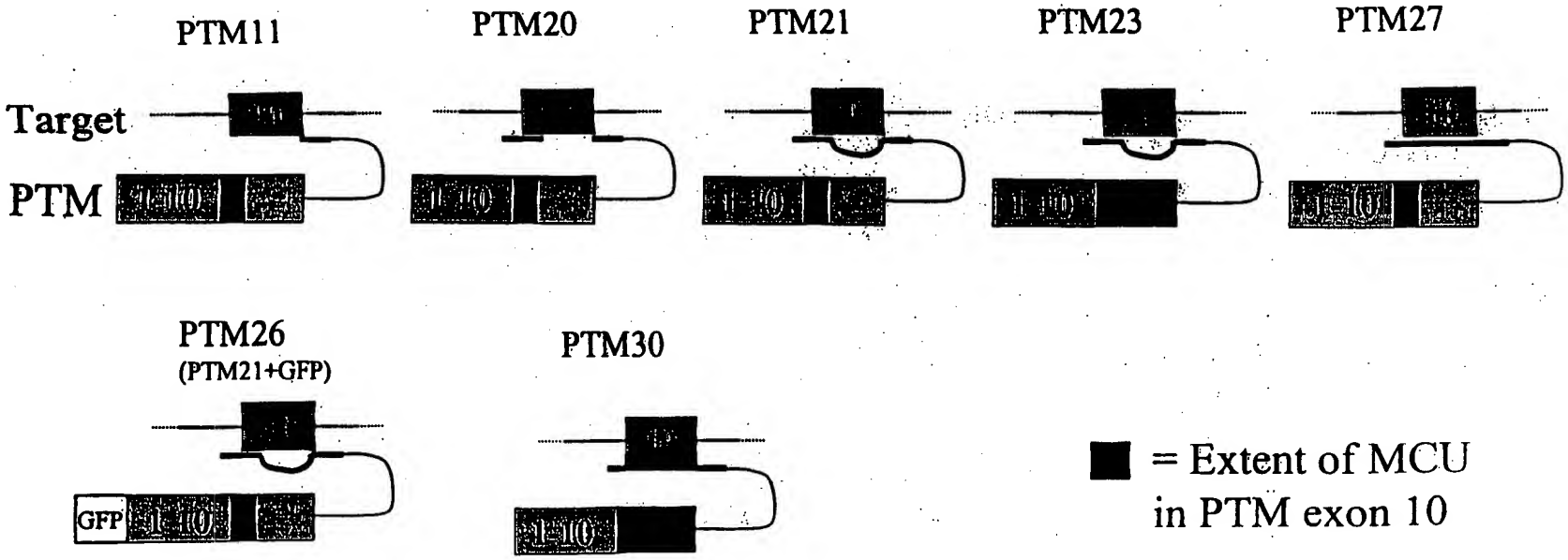
PTM with a long binding domain masking two splice sites in a mini-gene target.

C



PTM with a long binding domain masking two splice sites and the whole of exon 10 in a mini-gene target.

Figure 34



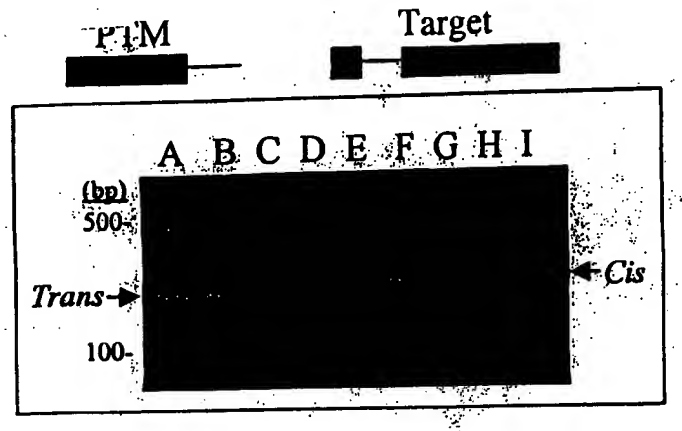
MCU in exon 10 of PTM

88 of 192 (46%) bases in PTM exon 10 are not complementary to its binding domain.

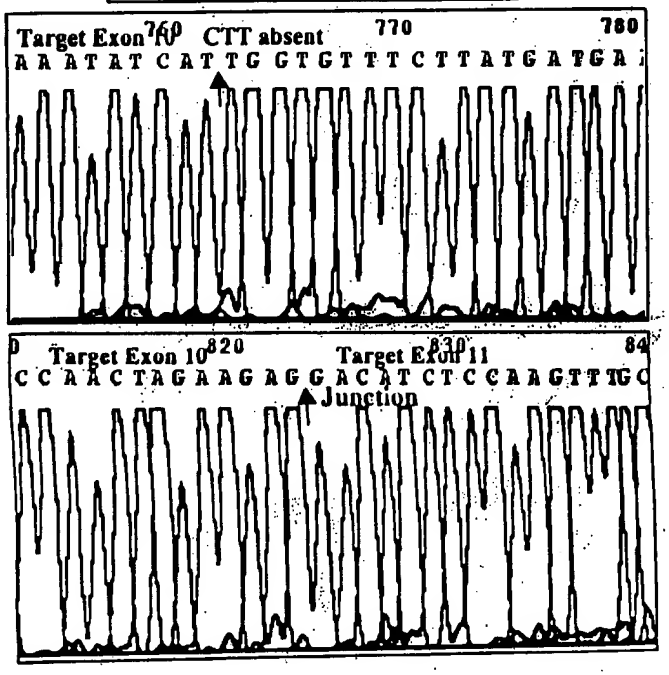
ACGAGCTTGCTCATGATGATCATGGGCGAGTTAGAACCAAGTGAAGGCAAGATCAAACATTCCG
GCCGCATCAGCTTTTGCAGCCAATTCAGTTGGATCATGCCCGGTACCATCAAGGAGAACATAAT
CTTCGGCGTCAGTTACGACGAGTACCGCTATCGCTCGGTGATTAAGGCCTGTCAGTTGGAGGAG

Figure 35

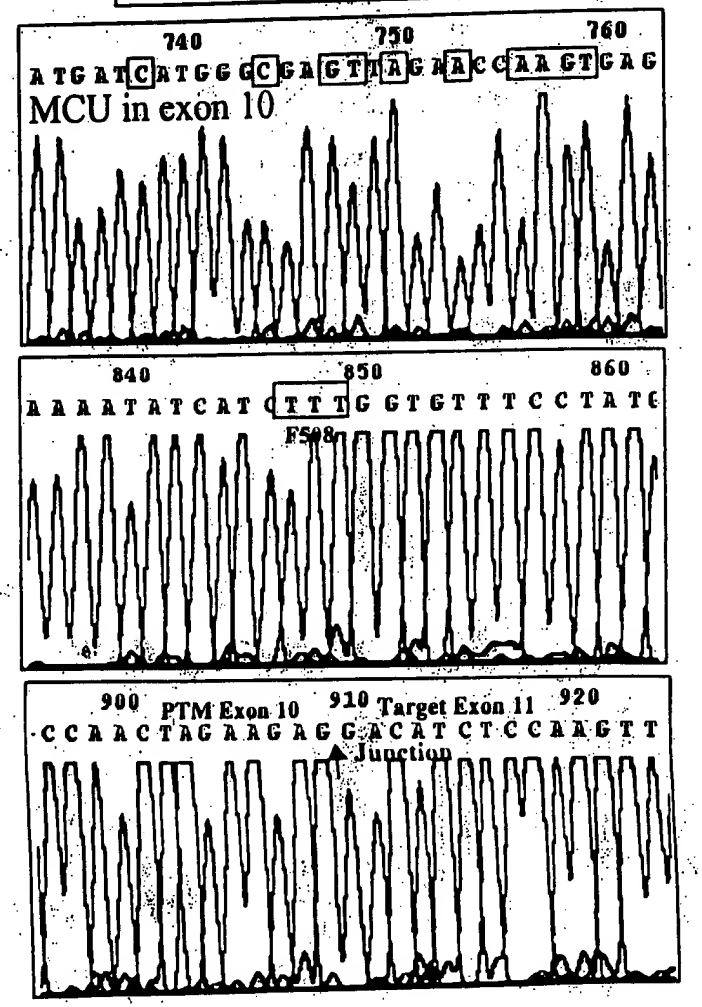
INTRONN



A. *Cis*-spliced product
[Primers CF1 + CF111]



B. *Trans*-spliced product
[Primers CF93 + CF111]



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5

Figure 36

A

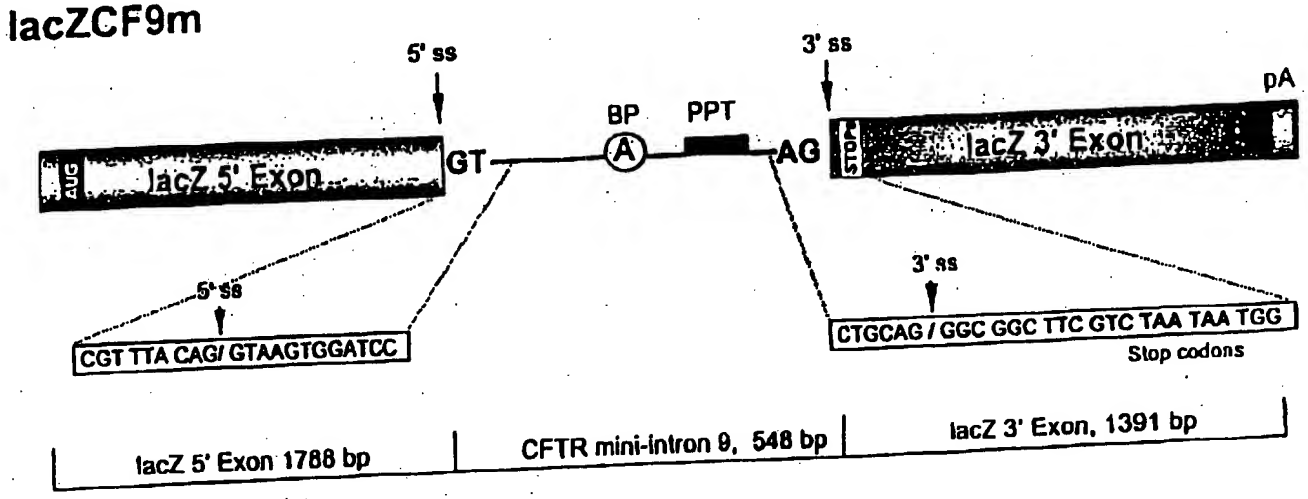
lacZCF9m
5' ss
3' ss
pA
BP
PPT
AUG
lacZ 5' Exon
GT
A
AG
STOP
lacZ 3' Exon
5' ss
CGT TTA CAG/ GTAAGTGGATCC
3' ss
CTGCAG / GGC GGC TTC GTC TAA TAA TGG
Stop codons
lacZ 5' Exon 1788 bp
CFTR mini-intron 9, 548 bp
lacZ 3' Exon, 1391 bp

Figure 37 A

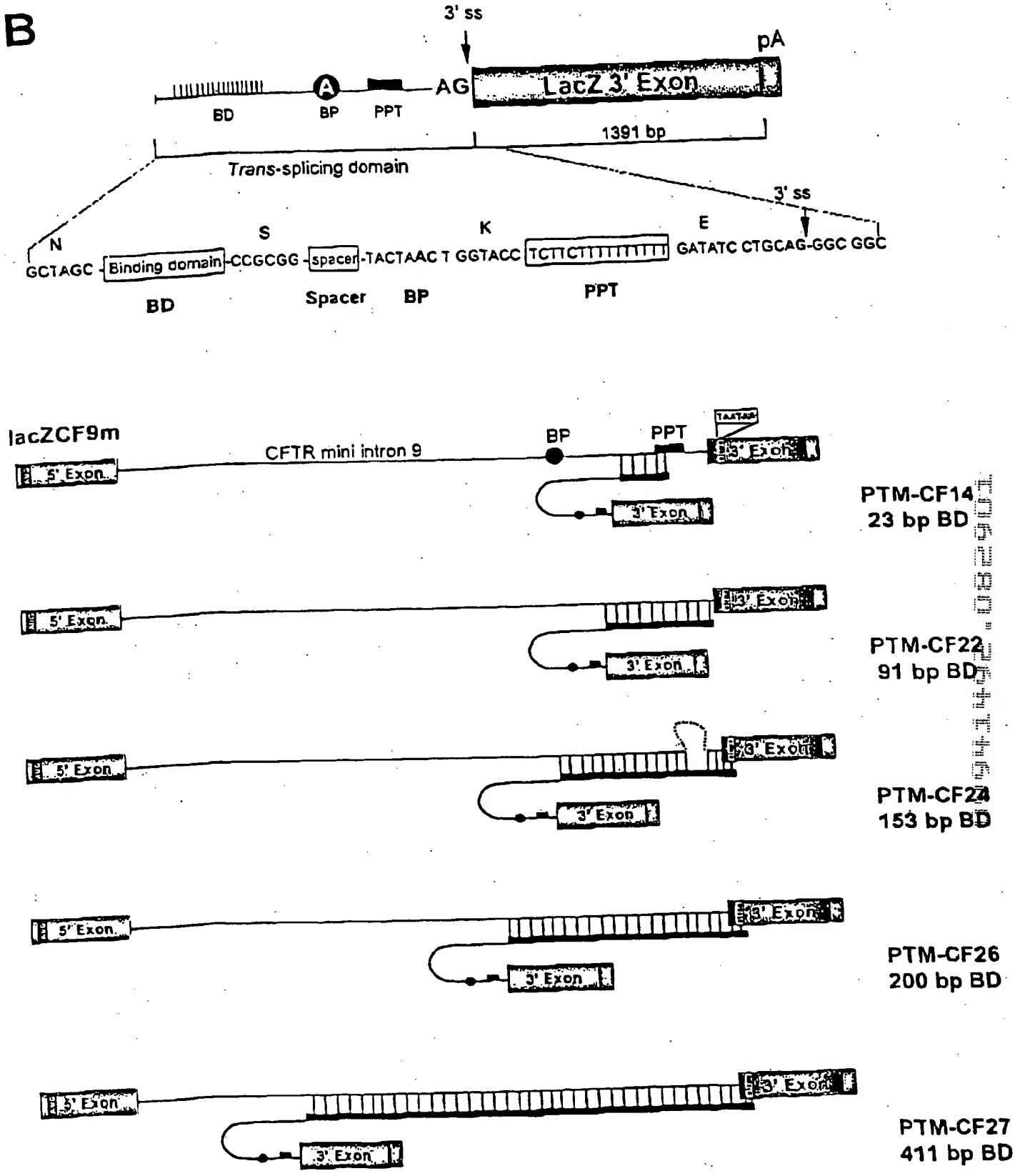
B
3' ss
pA
AG
LacZ 3' Exon
BD
BP
PPT
1391 bp
Trans-splicing domain
3' ss
N
S
K
E
GCTAGC
Binding domain
CCGCGG
spacer
TACTAAC T GGTACC
TCTTCTTTTTTTTT
GATATC CTGCAG-GGC GGC
BD
Spacer
BP
PPT
lacZCF9m
CFTR mini intron 9
BP
PPT
5' Exon
3' Exon
PTM-CF14
23 bp BD
PTM-CF22
91 bp BD
PTM-CF24
153 bp BD
PTM-CF26
200 bp BD
PTM-CF27
411 bp BD

Figure 37B

C

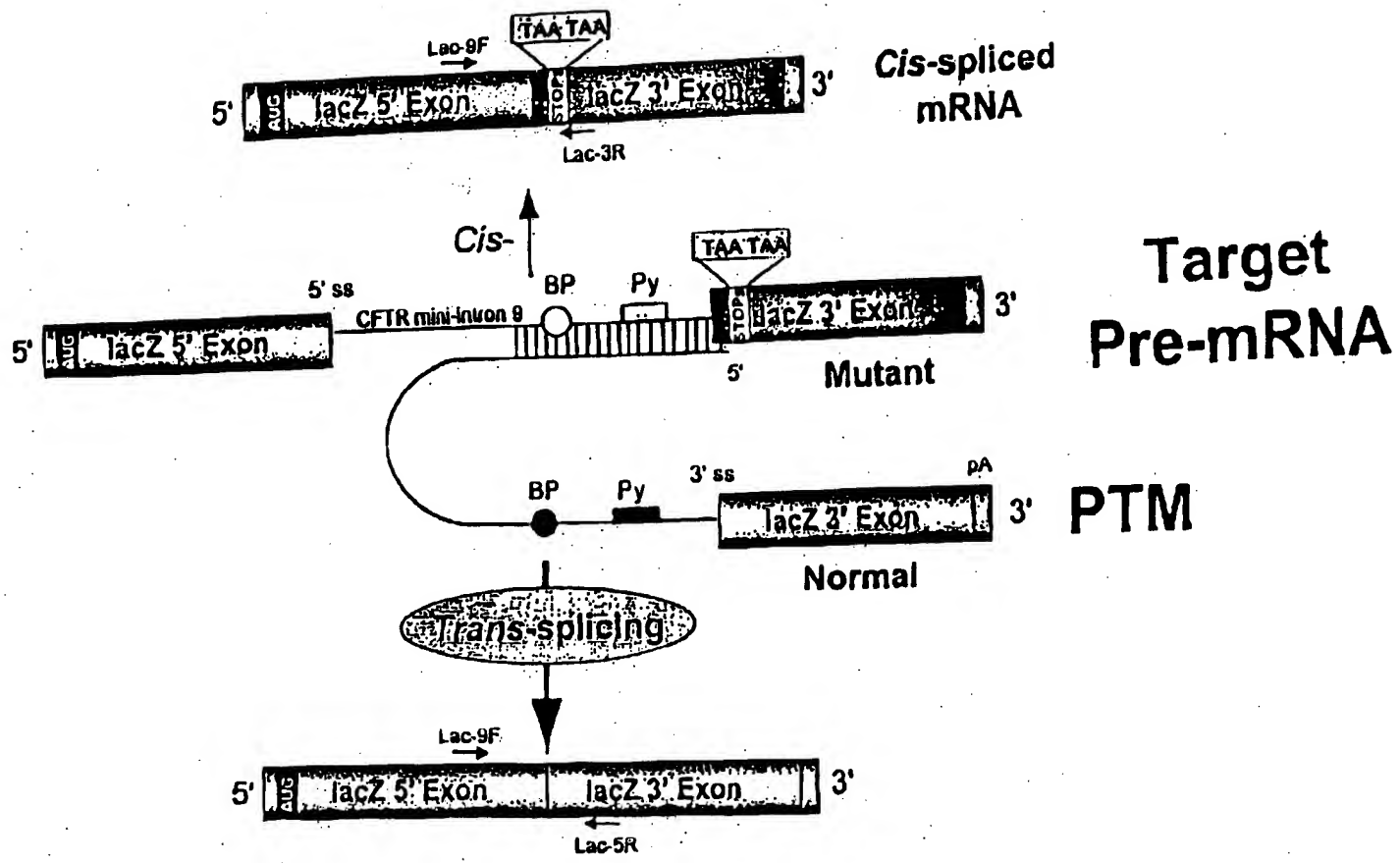

Figure 37C

A

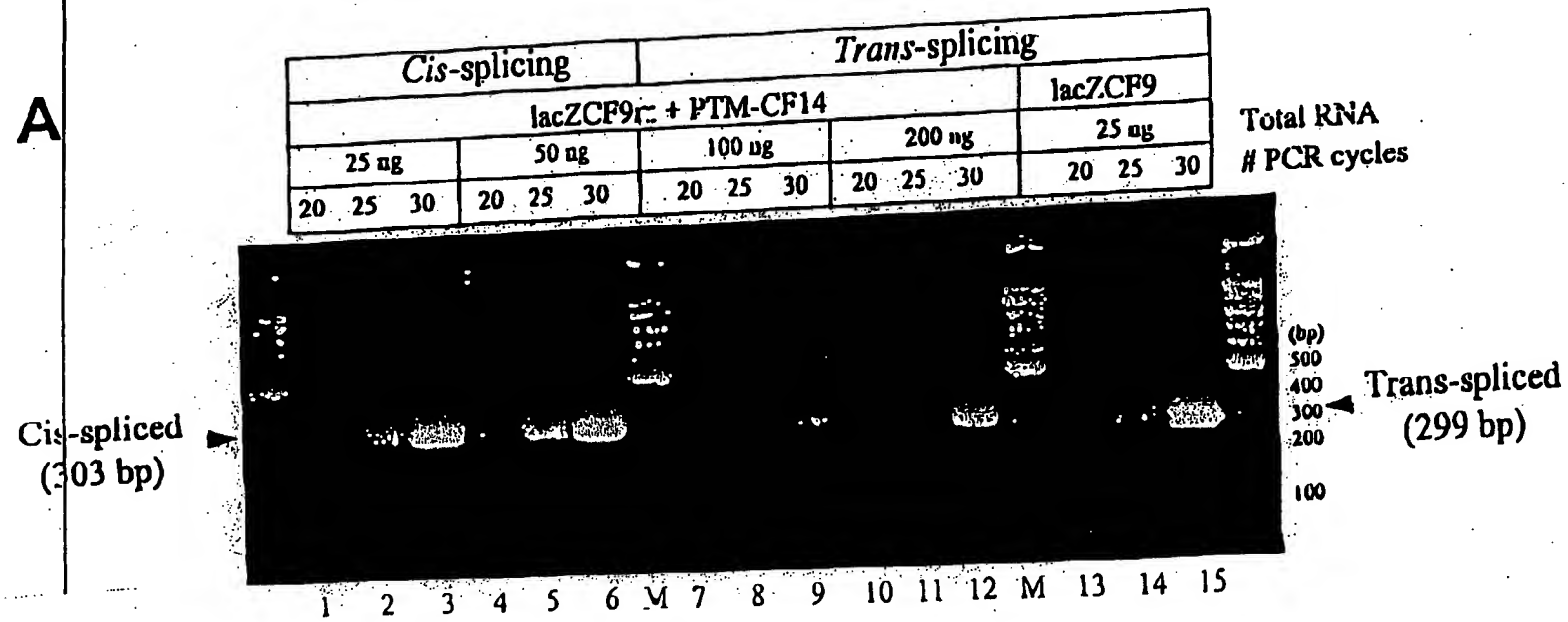

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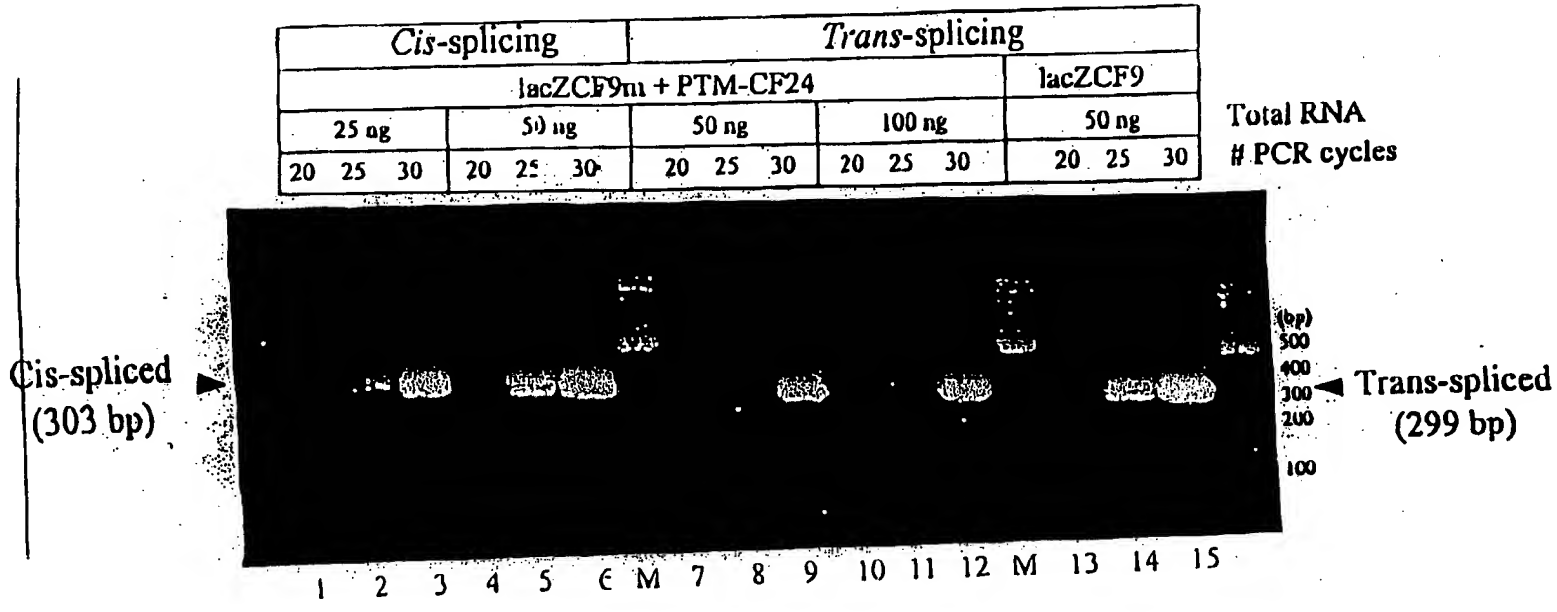
Figure 38 A



B

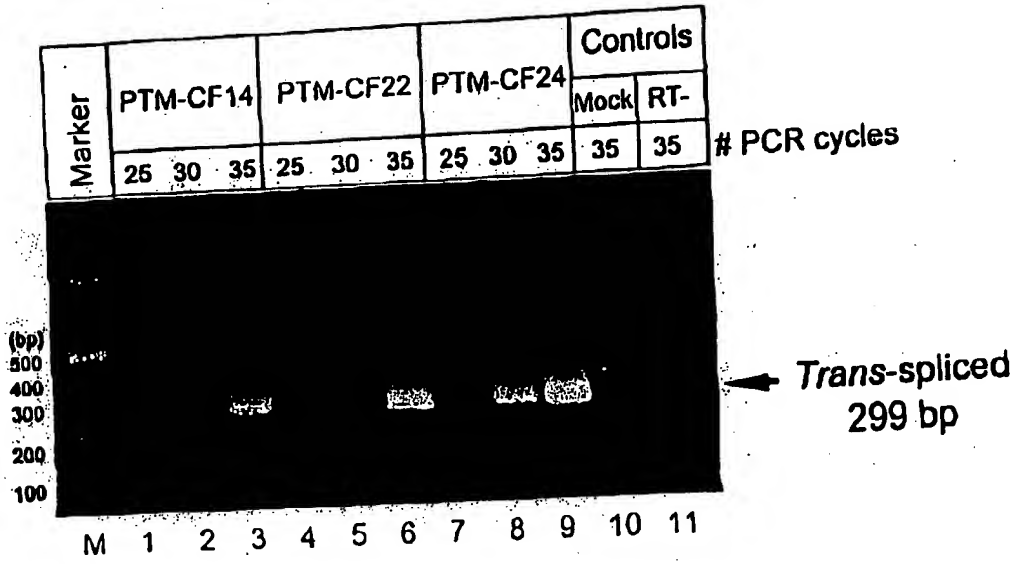


Figure 38B

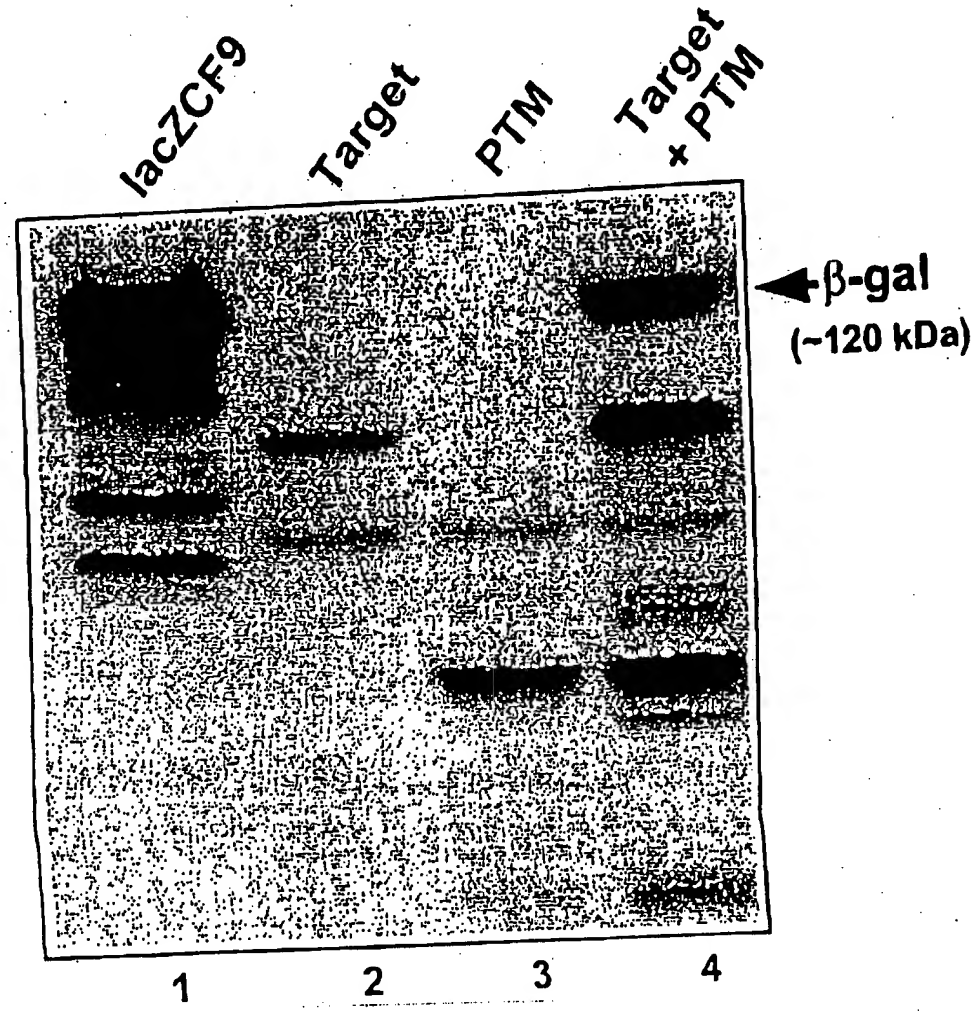


Figure 39

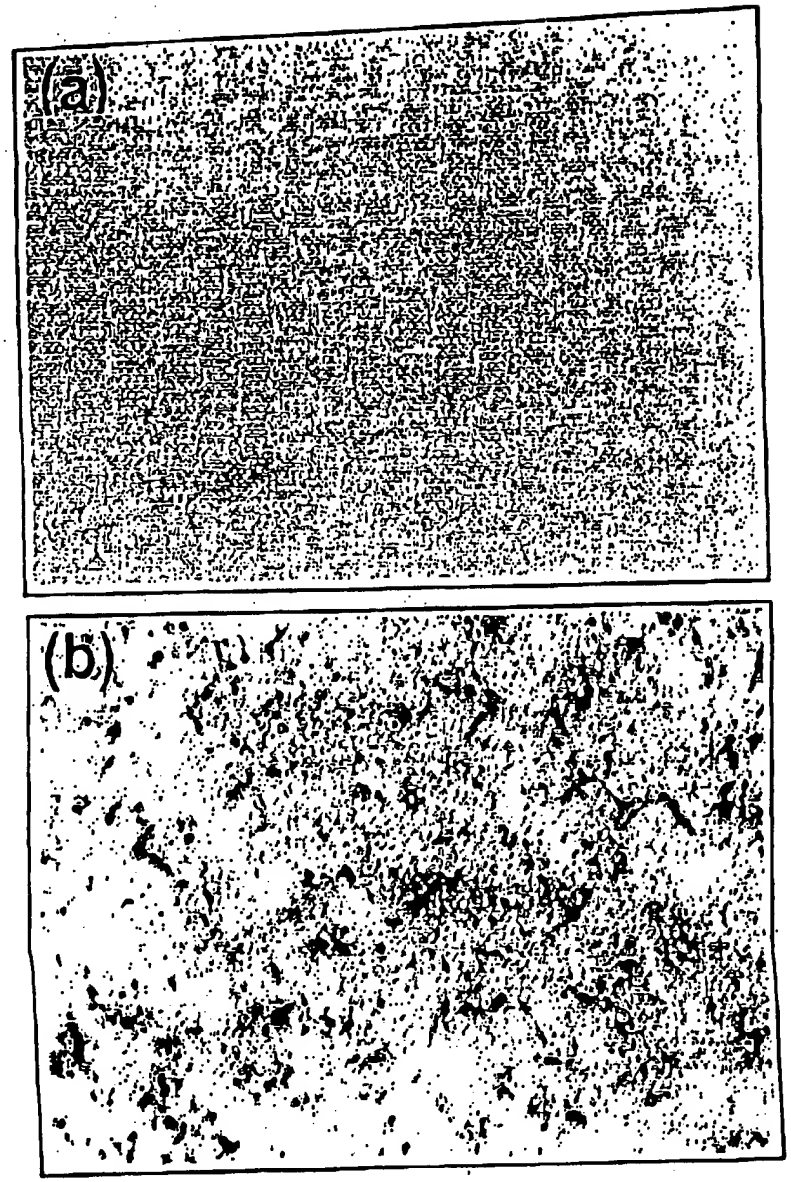


Figure 40A

B

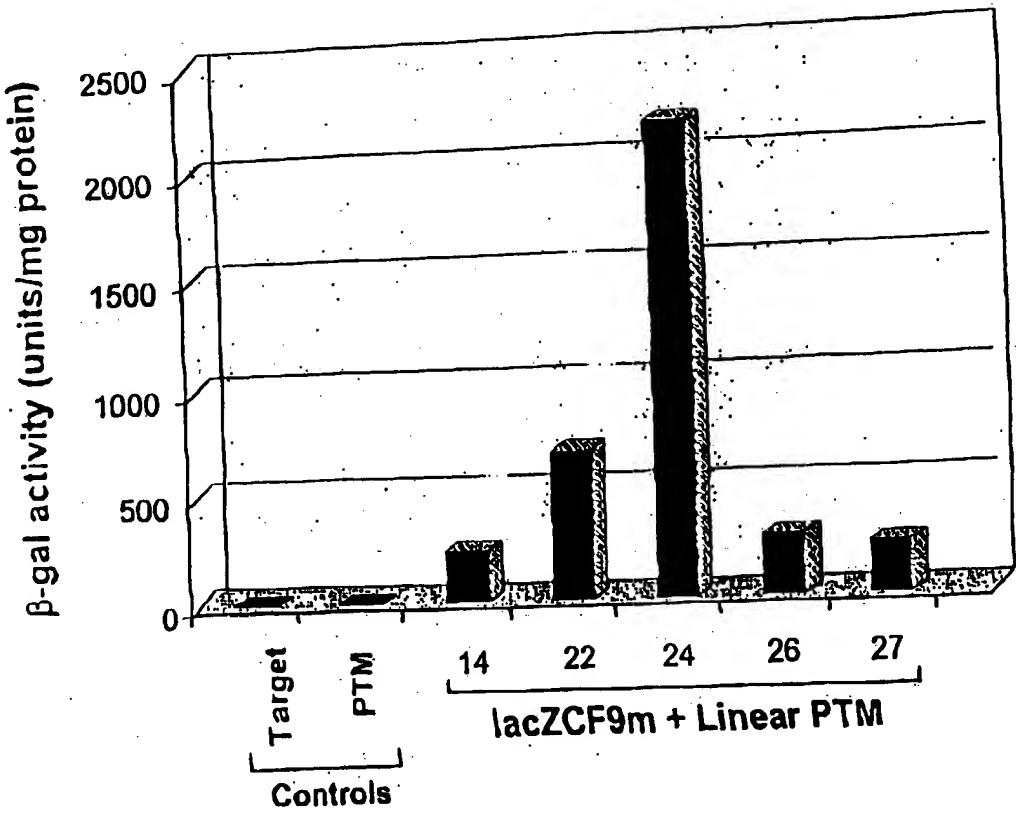


Figure 40B

C

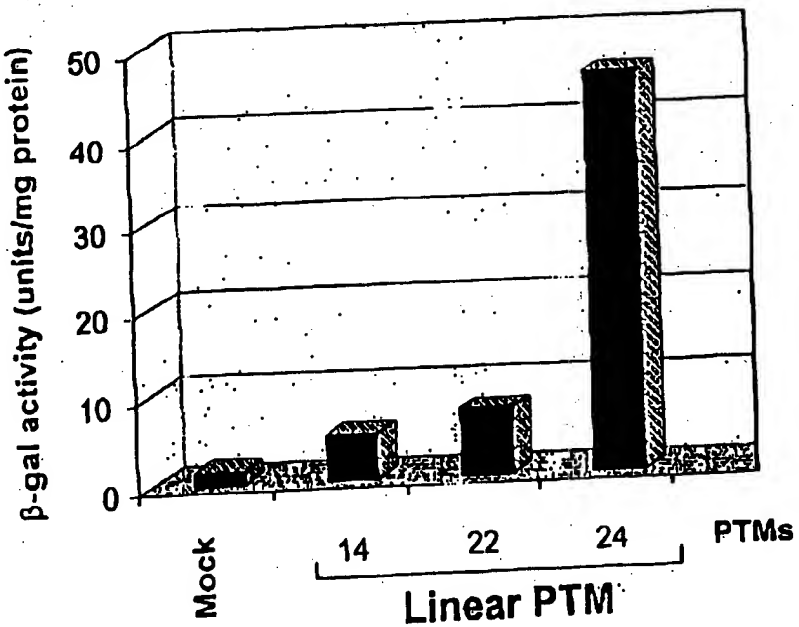

β-gal activity (units/mg protein)
50
40
30
20
10
0
Mock
14
22
24
PTMs
Linear PTM

Figure 40C

A

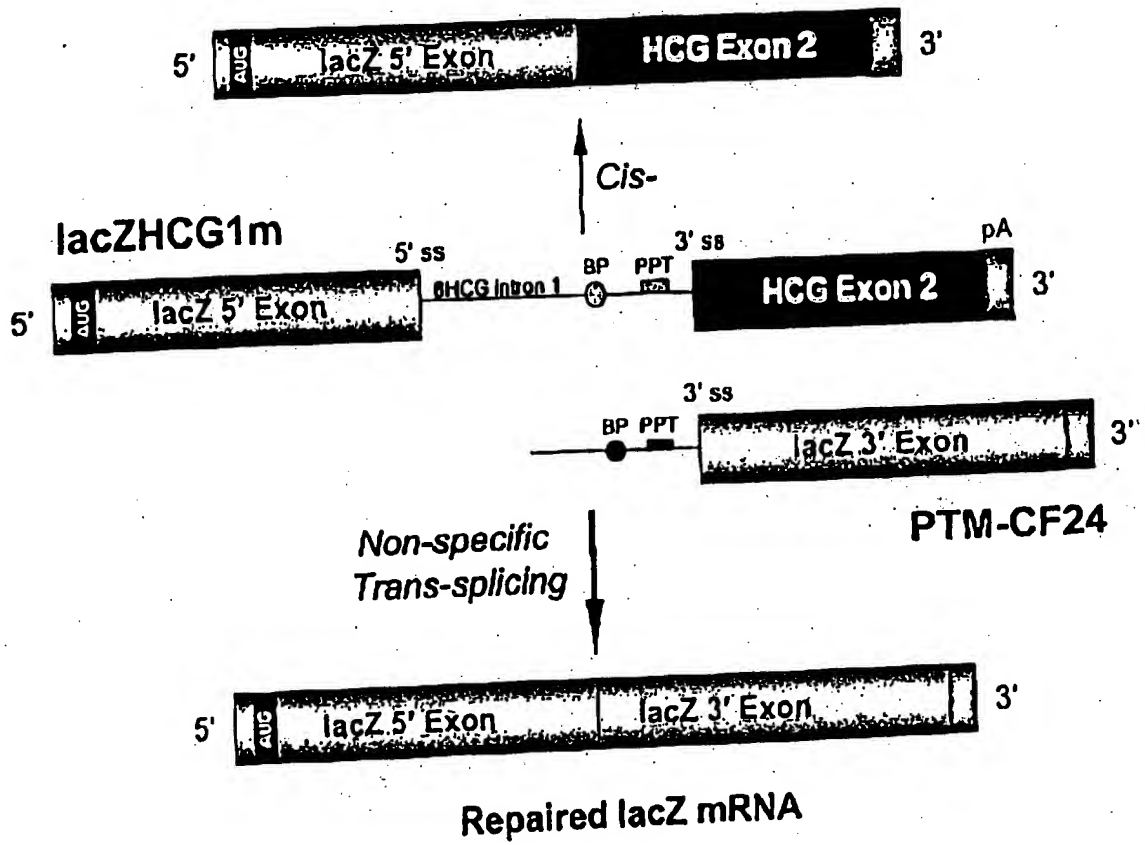


Figure 41A

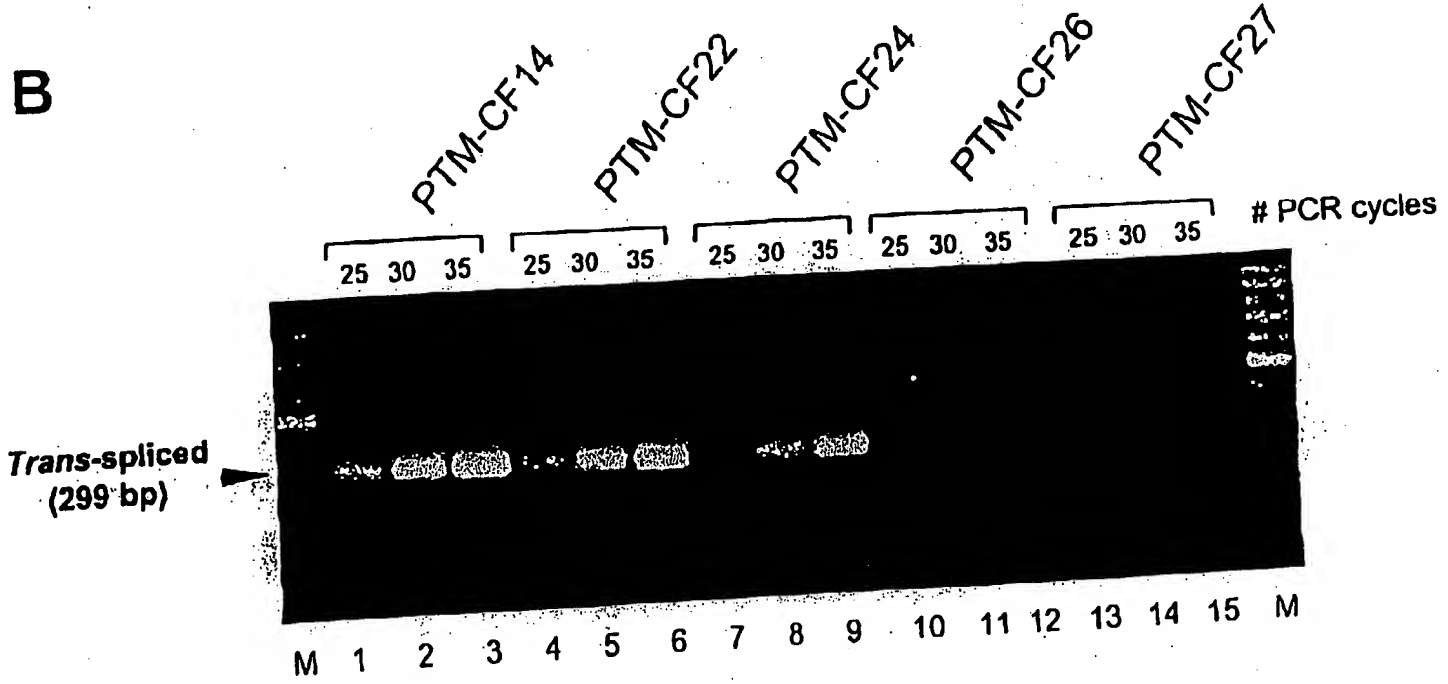

B
PTM-CF14
PTM-CF22
PTM-CF24
PTM-CF26
PTM-CF27
25 30 35
PCR cycles
Trans-spliced
(299 bp)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M

Figure 4B

C

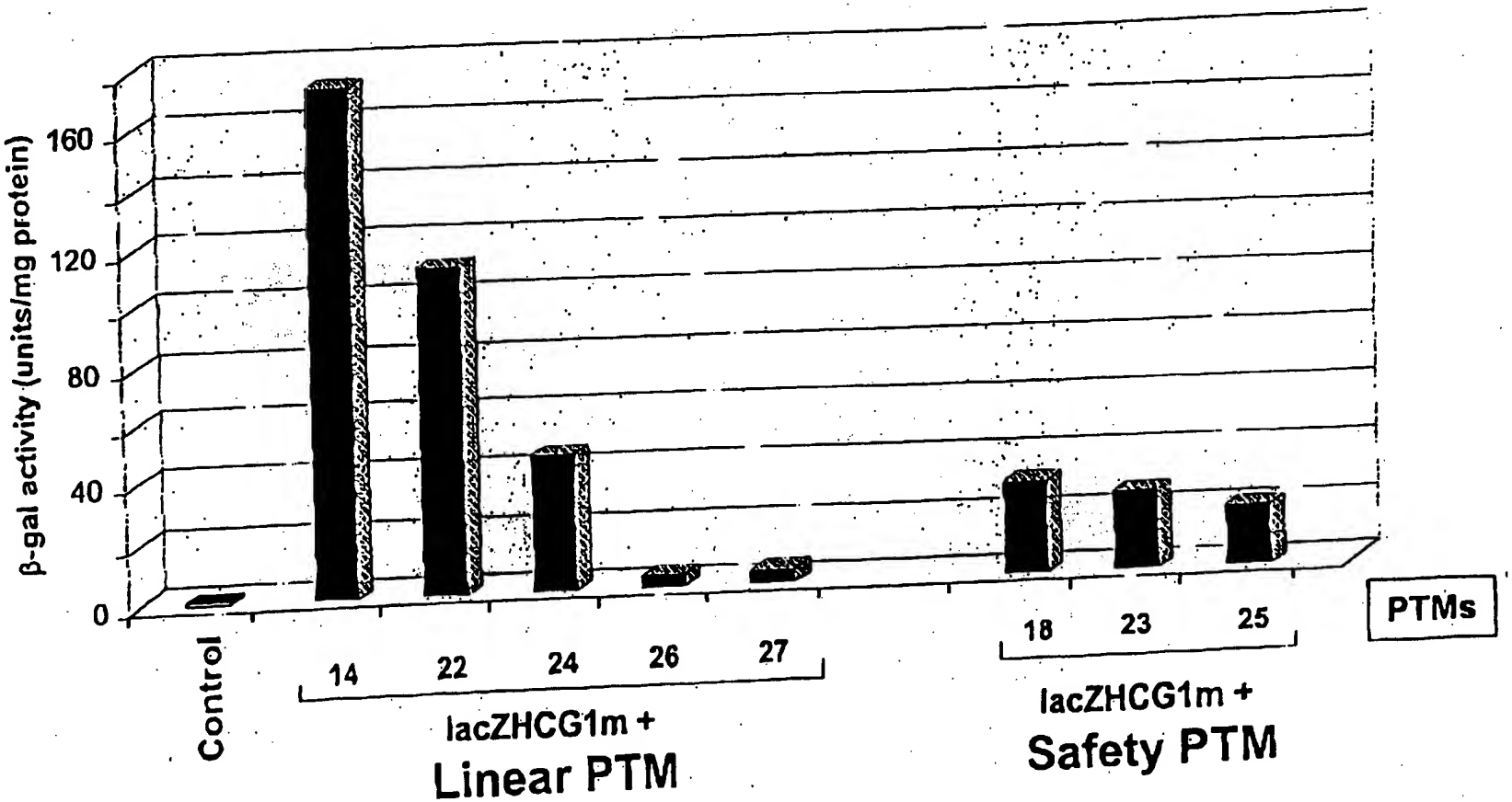

β-gal activity (units/mg protein)
160
120
80
40
0
Control
14
22
24
26
27
lacZHCG1m +
Linear PTM
18
23
25
PTMs
lacZHCG1m +
Safety PTM

Figure 41C

Exons 1-10

ATGCAGAGGTCGCCTCTGGAAAAGGCCAGCGTTGTCTCCAAACTTTTTTTCAGCTGGACCAGACCAATTTTGAGGAAAG
GATACAGACAGCGCCTGGAATTGTCAGACATATACCAAATCCCTTCTGTTGATTCTGCTGACAATCTATCTGAAAAATT
GGAAAGAGAATGGGATAGAGAGCTGGCTTCAAAGAAAAATCCTAAACTCATTAATGCCCTTCGGCGATGTTTTTTCTGG
AGATTTATGTTCTATGGAATCTTTTTATATTTAGGGGAAGTCACCAAAGCAGTACAGCCTCTCTTACTGGGAAGAATCA
TAGCTTCCTATGACCCGGATAACAAGGAGGAACGCTCTATCGCGATTTATCTAGGCATAGGCTTATGCCTTCTCTTTAT
TGTGAGGACACTGCTCCTACACCCAGCCATTTTTGGCCTTCATCACATTGGAATGCAGATGAGAATAGCTATGTTTAGT
TTGATTTATAAGAAGACTTTAAAGCTGTCAAGCCGTGTTCTAGATAAAATAAGTATTGGACAACTTGTTAGTCTCCTTT
CCAACAACCTGAACAAATTTGATGAAGGACTTGCATTGGCACATTTCGTGTGGATCGCTCCTTTGCAAGTGGCACTCCT
CATGGGGCTAATCTGGGAGTTGTTACAGGCGTCTGCCTTCTGTGGACTTGGTTTCCTGATAGTCCTTGCCCTTTTTCAG
GCTGGGCTAGGGAGAATGATGATGAAGTACAGAGATCAGAGAGCTGGGAAGATCAGTGAAAGACTTGTGATTACCTCAG
AAATGATCGAGAACATCCAATCTGTTAAGGCATACTGCTGGGAAGAAGCAATGGAAAAAATGATTGAAAACTTAAGACA
AACAGAACTGAAACTGACTCGGAAGGCAGCCTATGTGAGATACTTCAATAGCTCAGCCTTCTTCTTCTCAGGGTTCTTT
GTGGTGTTTTTATCTGTGCTTCCCTATGCACTAATCAAAGGAATCATCCTCCGGAAAATATTCACCACCATCTCATTCT
GCATTGTTCTGCGCATGGCGGTCACTCGGCAATTTCCCTGGGCTGTACAAACATGGTATGACTCTCTTGGAGCAATAAA
CAAAATACAGGATTTCTTACAAAAGCAAGAATATAAGACATTGGAATATAACTTAACGACTACAGAAGTAGTGATGGAG
AATGTAACAGCCTTCTGGGAGGAGGGATTTGGGGAATTATTTGAGAAAGCAAAACAAAACAATAACAATAGAAAAACTT
CTAATGGTGATGACAGCCTCTTCTTCAGTAATTTCTCACTTCTTGGTACTCCTGTCCTGAAAGATATTAATTTCAAGAT
AGAAAGAGGACAGTTGTTGGCGGTTGCTGGATCCACTGGAGCAGGCAAGACGAGCTTGCTCATGATGATCATGGGCGAG
TTAGAACCAAGTGAAGGCAAGATCAAACATTCCGGCCGCATCAGCTTTTGCAGCCAATTCAGTTGGATCATGCCCGGTA
CCATCAAGGAGAACATAATCTTCGGCGTCAGTTACGACGAGTACCGCTATCGCTCGGTGATTAAGGCCTGTCAGTTGGA
GGAG

Trans-splicing domain

GTAAGATATCACCGATATGTGTCTAACCTGATTCGGGCCTTCGATACGCTAAGATCCACCGG
TCAAAAAGTTTTCACATAATTTCTTACCTCTTCTTGAATTCATGCTTTGATGACGCTTCTGTATCTATATTCATCATTG
GAAACACCAATGATATTTTCTTTAATGGTGCCTGGCATAATCCTGGAAAACTGATAACACAATGAAATTCTTCCACTGT
GCTTAATTTTACCCTCTGAATTCTCCATTTCTCCCATAATCATCATTACAACTGAACTCTGGAAATAAAACCCATCATT
ATTAACTCATTATCAAATCACGCT

Figure 42

153 bp PTM24 Binding Domain:

Nhe I 153 bp BD underlined

GCTAGC-[redacted]GACGAAGCCGCCCCTCACGCTCAGGATTCACTTGCCTCCAATTATCATCCTAAGCAGAAGTGTATA

TTCTTATTTGTAAAGATTCTATTAACTCATTTGATTCAAAATATTTAAAATACTTCCTGTTTCACCTACTCTGCTATGC

Sac II

AC-**CCGCGG**

Figure 43A

Trans-splicing domain

AATAATGACGAAGCCGCCCCTCACGCTCAGGATTCACTTGCCCTCCAATTATCATCCTAAGCAGAAGTGTATATTCTTA
TTTGTAAAGATTCTATTAACTCATTTGATTCAAAATATTTAAAATACTTCCTGTTTCACCTACTCTGCTATGCACCCGC
GGAACATTATTATAACGTTGCTCGAATACTAACTGGTACCTCTTCTTTTTTTTTTGATATCCTGCAG

Exons 10-24

ACTTCACTTCTAATGATGATTATGGGAGAACTGGAGCCTTCAGAGGGTAAAATTAAGCACAGTGGAAGAATTTCATTCT
GTTCTCAGTTTTCCTGGATTATGCCTGGCACCATTAAAGAAAATATCATCTTTGGTGTTTCCTATGATGAATATAGATA
CAGAAGCGTCATCAAAGCATGCCAACTAGAAGAGGACATCTCCAAGTTTGCAGAGAAAGACAATATAGTTCTTGGAGAA
GGTGGAATCACACTGAGTGGAGGTCAACGAGCAAGAATTTCTTTAGCAAGAGCAGTATACAAAGATGCTGATTTGTATT
TATTAGACTCTCCTTTTGGATACCTAGATGTTTTAACAGAAAAAGAAATATTTGAAAGCTGTGTCTGTAAACTGATGGC
TAACAAAACTAGGATTTTGGTCACTTCTAAAATGGAACATTTAAAGAAAGCTGACAAAATATTAATTTTGCATGAAGGT
AGCAGCTATTTTTATGGGACATTTTCAGAACTCCAAAATCTACAGCCAGACTTTAGCTCAAAACTCATGGGATGTGATT
CTTTCGACCAATTTAGTGCAGAAAGAAGAAATTCAATCCTAACTGAGACCTTACACCGTTTCTCATTAGAAGGAGATGC
TCCTGTCTCCTGGACAGAAACAAAAAAACAATCTTTTAAACAGACTGGAGAGTTTGGGGAAAAAAAGGAAGAATTCTATT
CTCAATCCAATCAACTCTATACGAAAATTTTCCATTGTGCAAAAGACTCCCTTACAAATGAATGGCATCGAAGAGGATT
CTGATGAGCCTTTAGAGAGAAGGCTGTCCTTAGTACCAGATTCTGAGCAGGGAGAGGCGATACTGCCTCGCATCAGCGT
GATCAGCACTGGCCCCACGCTTCAGGCACGAAGGAGGCAGTCTGTCCTGAACCTGATGACACACTCAGTTAACCAAGGT
CAGAACATTCACCGAAAGACAACAGCATCCACACGAAAAGTGTCACTGGCCCCTCAGGCAAACTTGACTGAACTGGATA
TATATTCAAGAAGGTTATCTCAAGAAACTGGCTTGGAAATAAGTGAAGAAATTAACGAAGAAGACTTAAAGGAGTGCTT
TTTTGATGATATGGAGAGCATACCAGCAGTGACTACATGGAACACATACCTTCGATATATTACTGTCCACAAGAGCTTA
ATTTTTGTGCTAATTTGGTGCTTAGTAATTTTTCTGGCAGAGGTGGCTGCTTCTTTGGTTGTGCTGTGGCTCCTTGGAA
ACACTCCTCTTCAAGACAAAGGGAATAGTACTCATAGTAGAAATAACAGCTATGCAGTGATTATCACCAGCACCAGTTC
GTATTATGTGTTTTACATTTACGTGGGAGTAGCCGACACTTTGCTTGCTATGGGATTCTTCAGAGGTCTACCACTGGTG
CATACTCTAATCACAGTGTCGAAAATTTTACACCACAAAATGTTACATTCTGTTCTTCAAGCACCTATGTCAACCCTCA
ACACGTTGAAAGCAGGTGGGATTCTTAATAGATTCTCCAAAGATATAGCAATTTTGGATGACCTTCTGCCTCTTACCAT
ATTTGACTTCATCCAGTTGTTATTAATTGTGATTGGAGCTATAGCAGTTGTCGCAGTTTTACAACCCTACATCTTTGTT
GCAACAGTGCCAGTGATAGTGGCTTTTATTATGTTGAGAGCATATTTCCTCCAAACCTCACAGCAACTCAAACAACTGG
AATCTGAAGGCAGGAGTCCAATTTTCACTCATCTTGTTACAAGCTTAAAAGGACTATGGACACTTCGTGCCTTCGGACG
GCAGCCTTACTTTGAAACTCTGTTCCACAAAGCTCTGAATTTACATACTGCCAACTGGTTCTTGTACCTGTCAACACTG
CGCTGGTTCCAAATGAGAATAGAAATGATTTTTGTCATCTTCTTCATTGCTGTTACCTTCATTTCCATTTTAACAACAG
GAGAAGGAGAAGGAAGAGTTGGTATTATCCTGACTTTAGCCATGAATATCATGAGTACATTGCAGTGGGCTGTAAACTC
CAGCATAGATGTGGATAGCTTGATGCGATCTGTGAGCCGAGTCTTTAAGTTCATTGACATGCCAACAGAAGGTAAACCT
ACCAAGTCAACCAAACCATACAAGAATGGCCAACTCTCGAAAGTTATGATTATTGAGAATTCACACGTGAAGAAAGATG
ACATCTGGCCCTCAGGGGGCCAAATGACTGTCAAAGATCTCACAGCAAAATACACAGAAGGTGGAAATGCCATATTAGA
GAACATTTCCTTCTCAATAAGTCCTGGCCAGAGGGTGGGCCTCTTGGGAAGAACTGGATCAGGGAAGAGTACTTTGTTA
TCAGCTTTTTTGAGACTACTGAACACTGAAGGAGAAATCCAGATCGATGGTGTGTCTTGGGATTCAATAACTTTGCAAC
AGTGGAGGAAAGCCTTTGGAGTGATACCACAGAAAGTATTTATTTTTTCTGGAACATTTAGAAAAAACTTGGATCCCTA
TGAACAGTGGAGTGATCAAGAAATATGGAAAGTTGCAGATGAGGTTGGGCTCAGATCTGTGATAGAACAGTTTCCTGGG
AAGCTTGACTTTGTCCTTGTGGATGGGGGCTGTGTCCTAAGCCATGGCCACAAGCAGTTGATGTGCTTGGCTAGATCTG
TTCTCAGTAAGGCGAAGATCTTGCTGCTTGATGAACCCAGTGCTCATTTGGATCCAGTAACATACCAAATAATTAGAAG
AACTCTAAAACAAGCATTTGCTGATTGCACAGTAATTCTCTGTGAACACAGGATAGAAGCAATGCTGGAATGCCAACAA
TTTTTGGTCATAGAAGAGAACAAAGTGCGGCAGTACGATTCCATCCAGAAACTGCTGAACGAGAGGAGCCTCTTCCGGC
AAGCCATCAGCCCCTCCGACAGGGTGAAGCTCTTTCCCCACCGGAACTCAAGCAAGTGCAAGTCTAAGCCCCAGATTGC

Histidine tag Stop

TGCTCTGAAAGAGGAGACAGAAGAAGAGGTGCAAGATACAAGGCTTCATCATCATCATCATTAG

Figure 43B

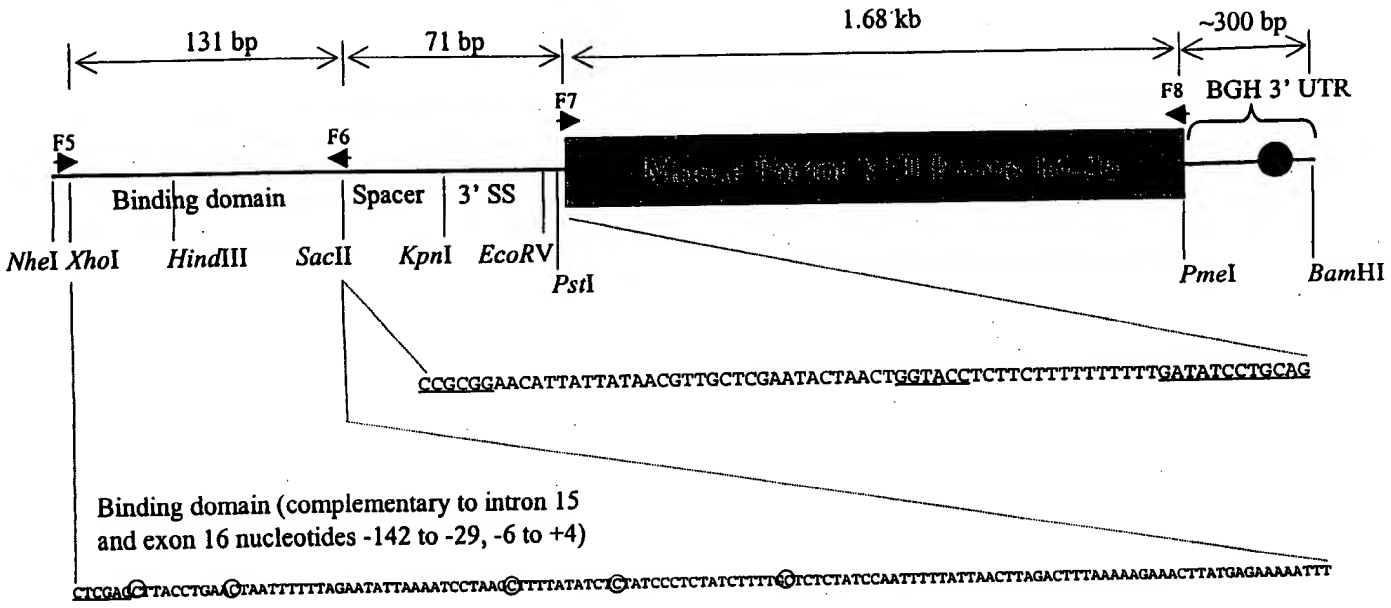


Figure 44 A

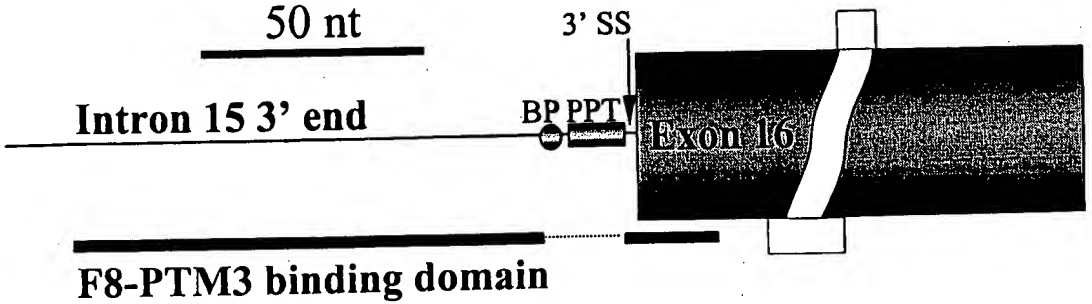
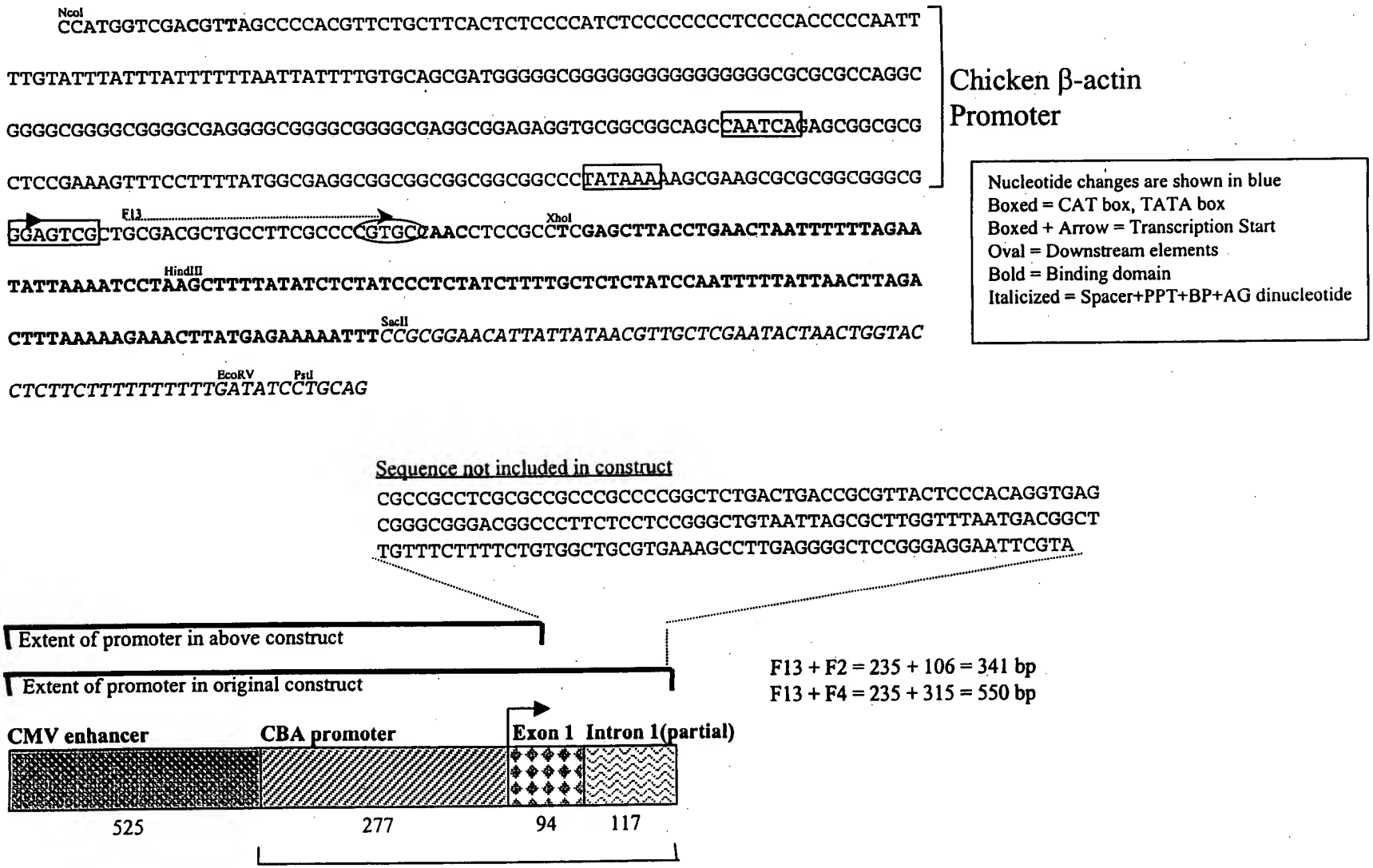
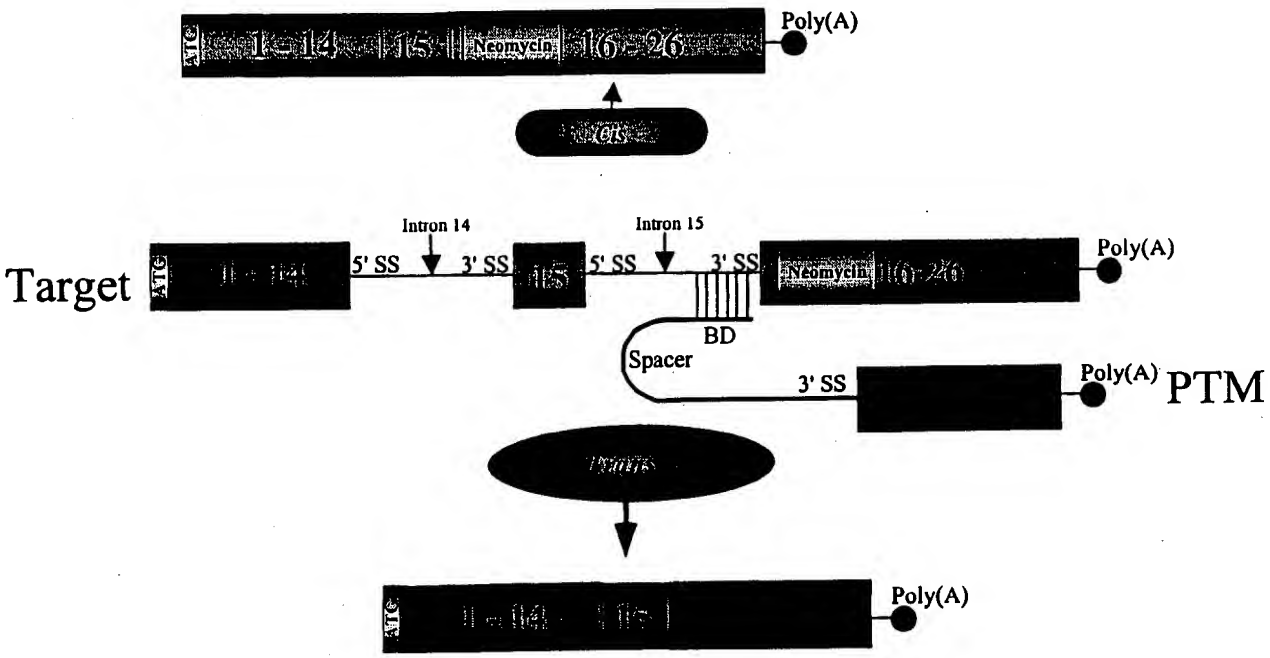


Figure 44 B

Figure 44C



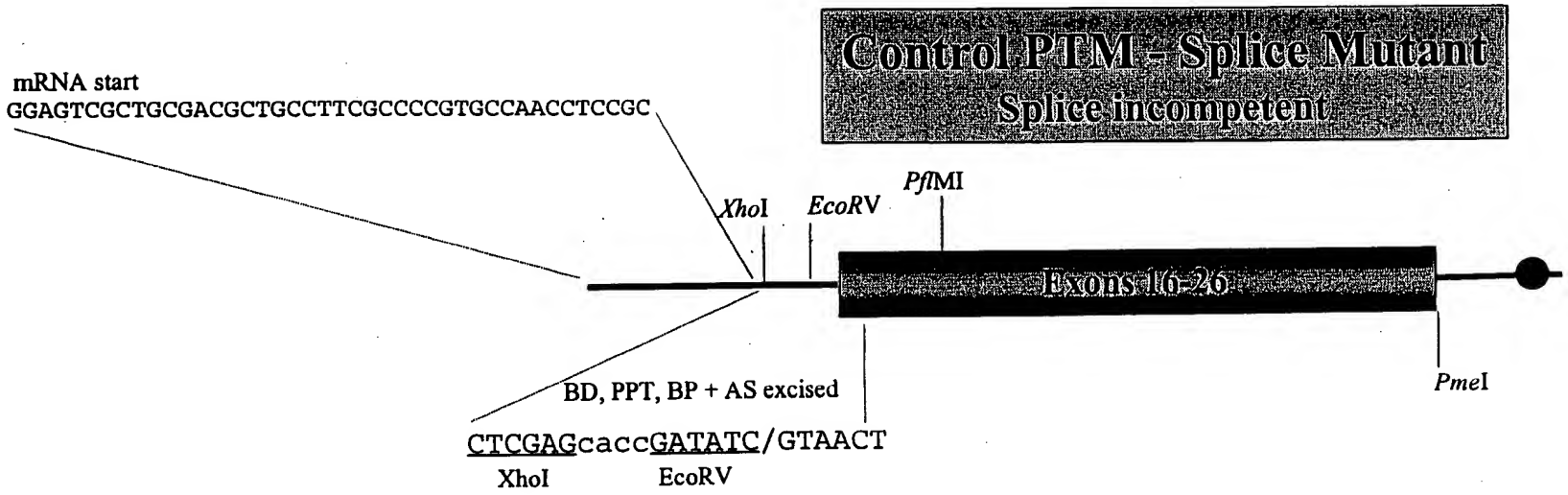
Chicken Beta Actin Promoter (including exon 1 and part of intron 1)



Repaired full length Factor VIII mRNA

Figure 44D

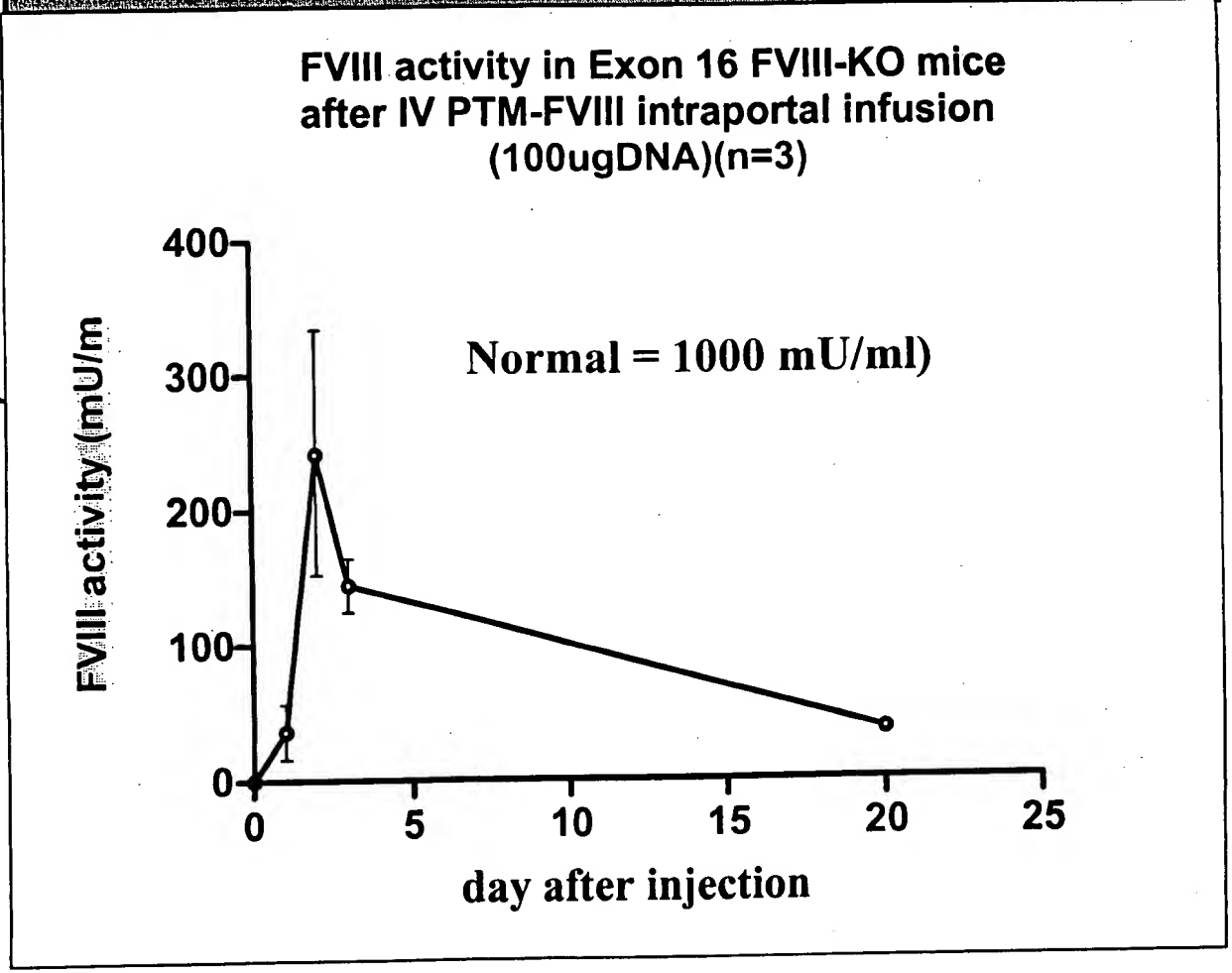
Figure 45



Method:
Excise TSD and part of exon 16 with
XhoI and PflMI and ligate in a PCR product that:
1) eliminates the TSD and splice acceptor site
2) inserts EcoRV adjacent to exon 16
3) restores the coding for exon 16

Repair of Factor VIII

Preliminary results from one experiment



METHODS

Inject plasmid intraportally

↓

Sample blood (1, 2, 3, 20 d)

↓

Assay for factor VIII activity

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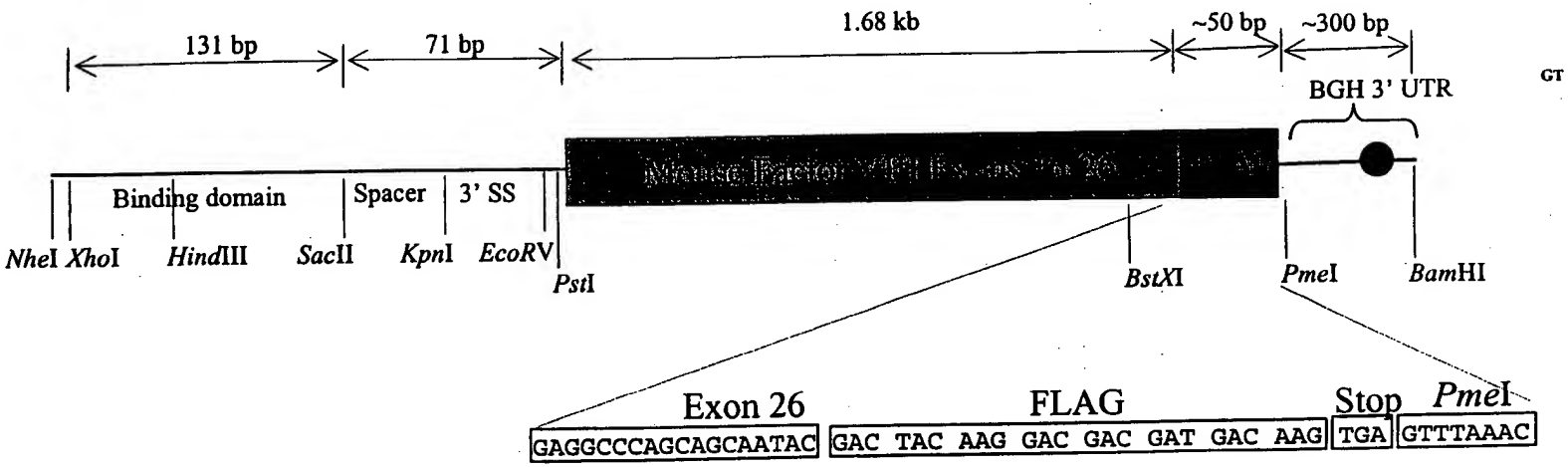
Figure 46

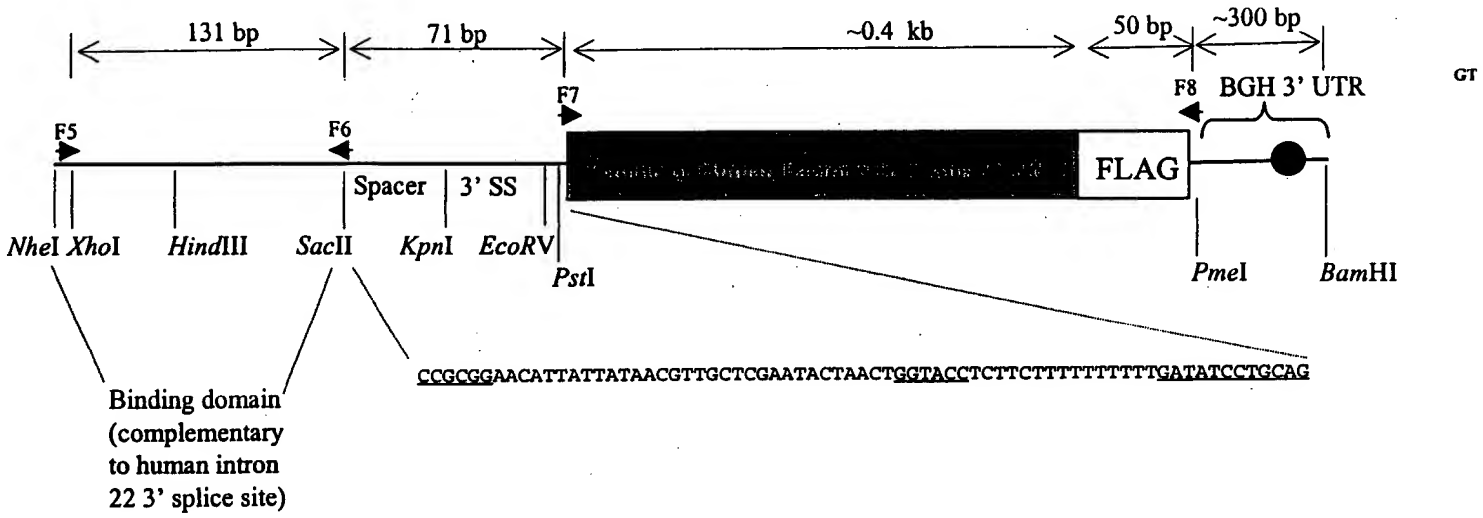
Detailed structure of a mouse factor VIII PTM containing normal sequences for exons 16-26 and a C-terminal FLAG tag. BGH = bovine growth hormone 3' UTR; Binding domain = 125 bp.



REFERENCE FOR DESIGN OF FLAG TAG

Brann T, Kayda D, Lyons RM, Shirley P, Roy S, Kaleko M, Smith T.
Adenoviral vector-mediated expression of physiologic levels of human factor VIII in nonhuman primates.
Hum Gene Ther 1999 Dec 10;10(18):2999-3011
Genetic Therapy, Inc., a Novartis Company, Gaithersburg, MD 20878, USA.
Epitope-tagged B domain-deleted human factor VIII cDNA (flagged FVIII) was evaluated in nonhuman primates.

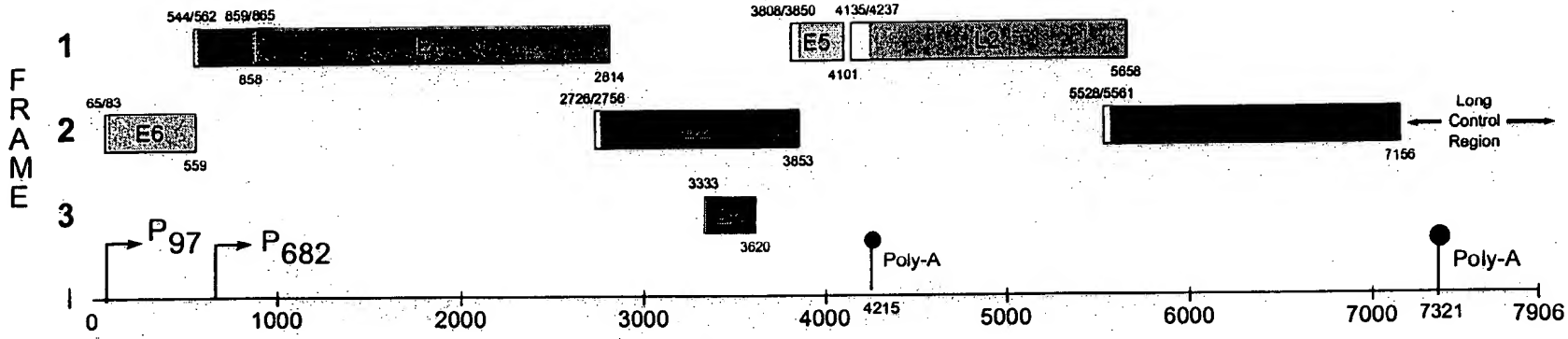
Figure 47A

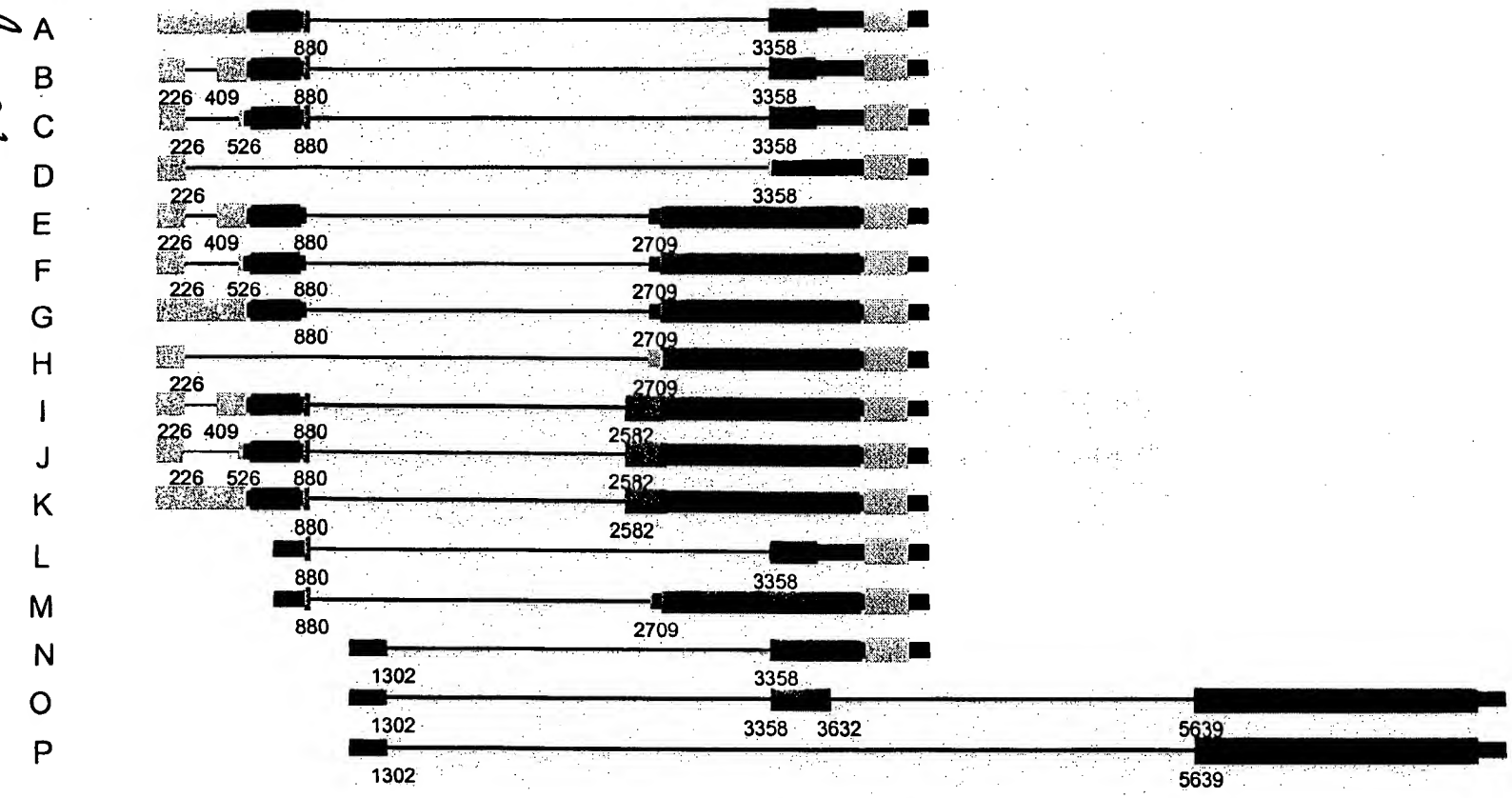


FLAG = C-terminal tag to be used to detect repaired factor VIII protein.

Figure 47B

Transcription Map of HPV-16





Coding Potential

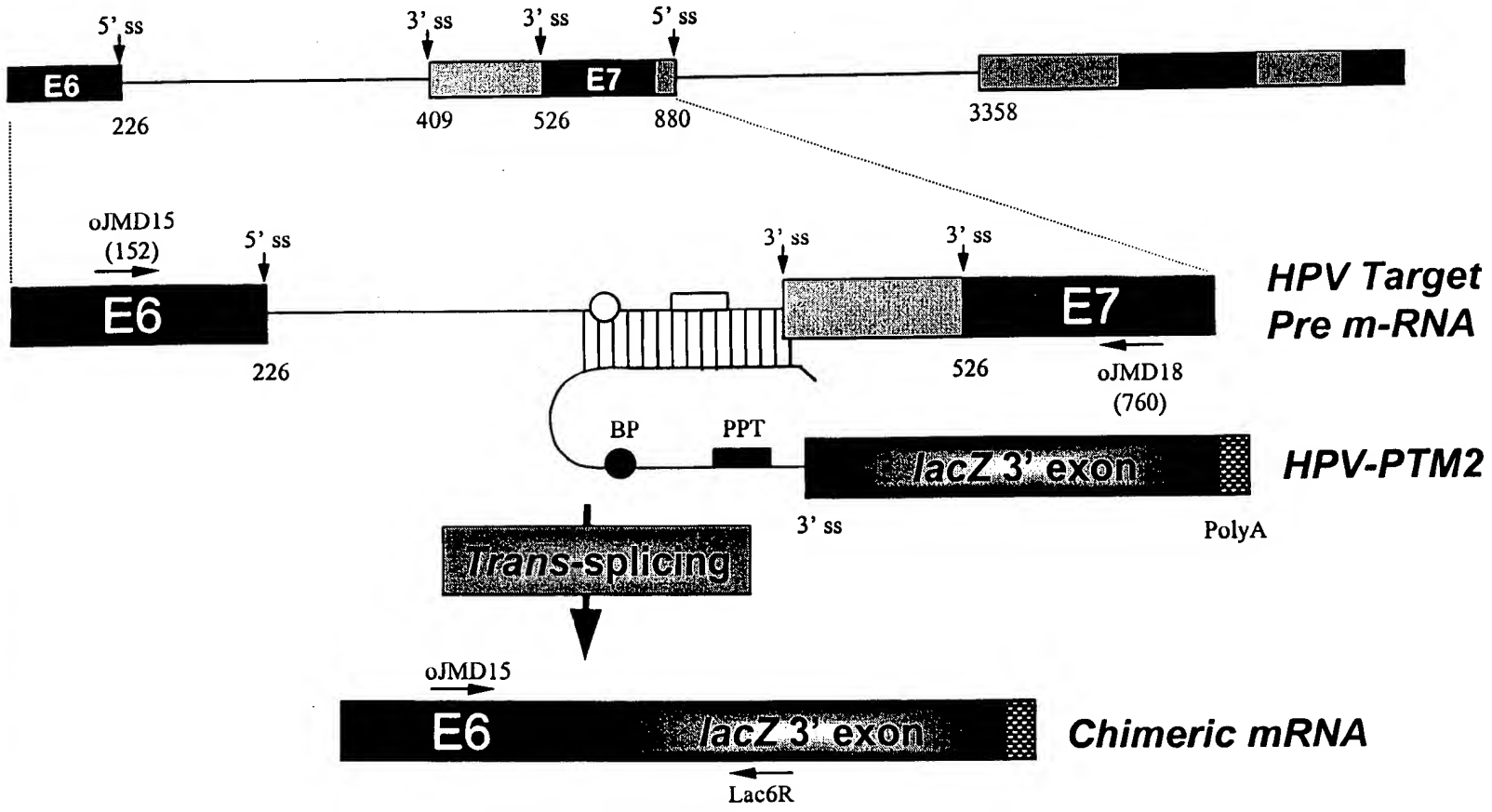
- E6,E7,E1^E4,E5
- E6*I,E7,E1^E4,E5
- E6*II,E7,E1^E4,E5
- E6*III,E5
- E6*I,E7,E2,E5
- E6*II,E7,E2,E5
- E6,E7,E2,E5
- E6*IV,E2,E5
- E6*I,E7,E1C,E2,E5
- E6*II,E7,E1C,E2,E5
- E6,E7,E1C,E2,E5
- E1^E4,E5
- E1*,E2,E5
- E2C,E5
- E2M,L1
- L1

INTRONN

FIGURE 48

SMaRT Strategy to Disrupt the Expression of Human Papillomavirus Type 16

Targeting E6 Exon



SMaRT Strategy by 3' Exon Replacement: **Schematic diagram of HPV-PTM2 binding to the 3' splice site of the HPV type 16 target pre-mRNA**

FIGURE 49

Targeting 880 Splice Donor Site

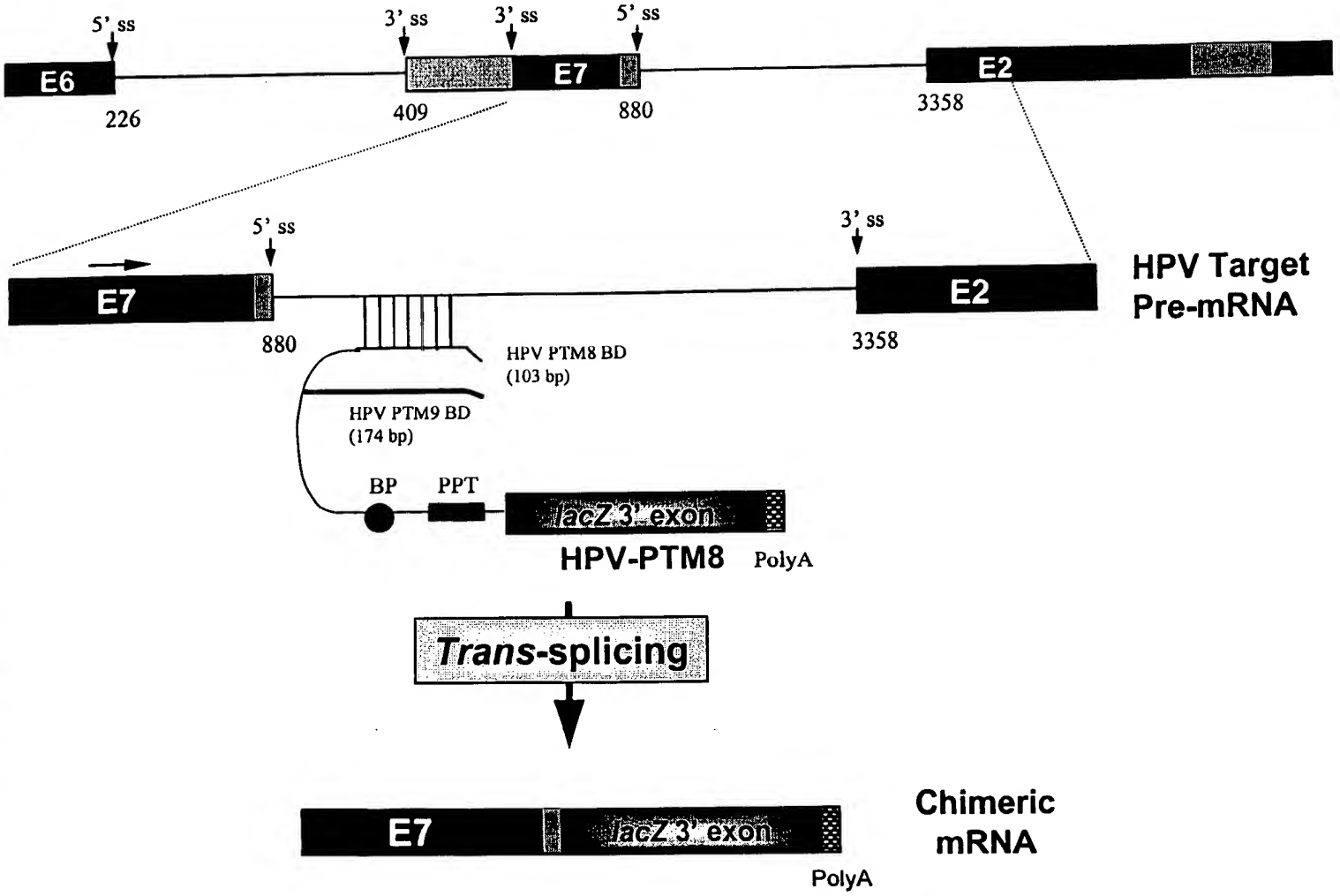


FIGURE 50

INTRONN

PTM Design

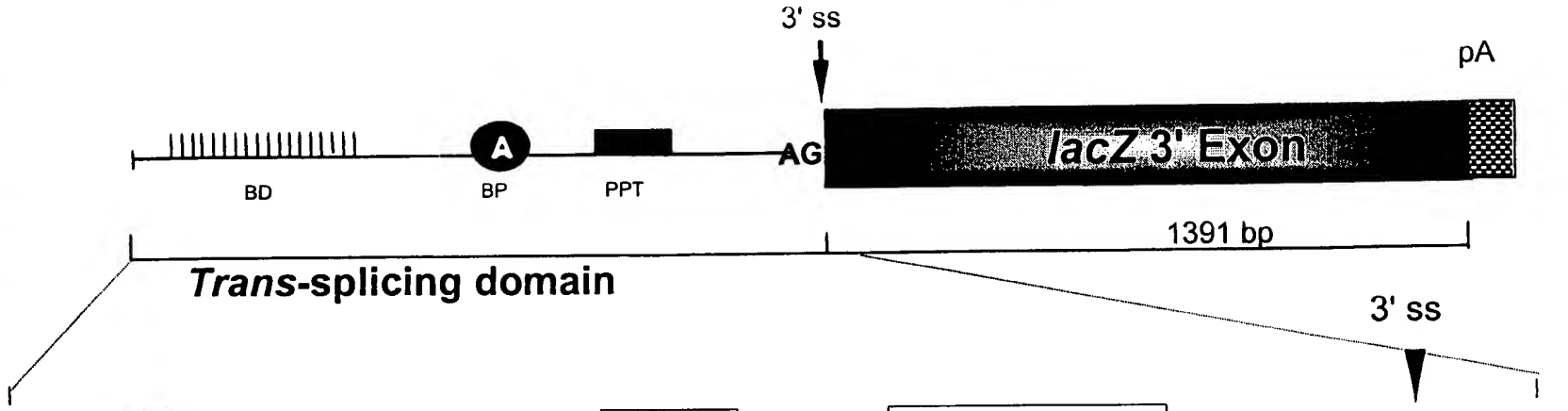
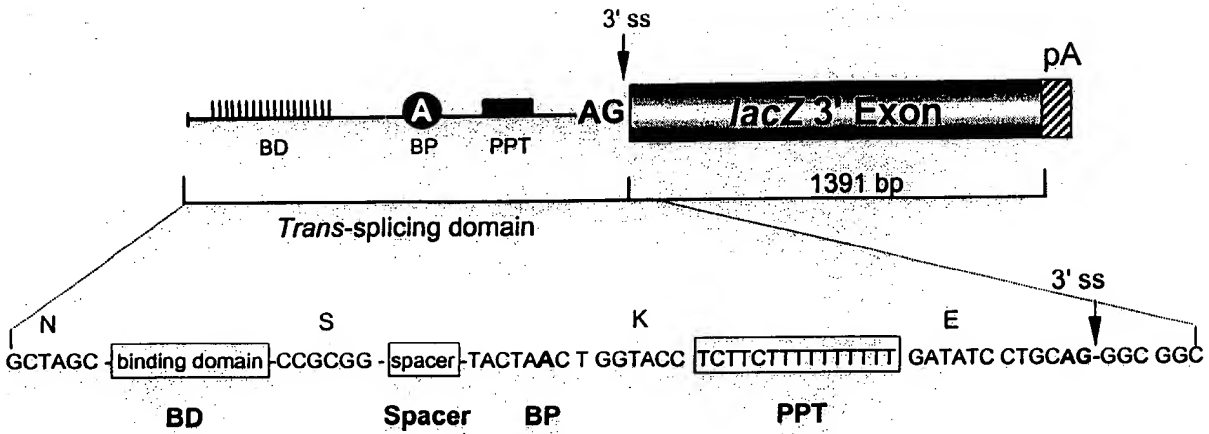


FIGURE 51

INTRONN

HPV-PTM1 with 80 bp binding domain targeted to 3' ss at 409 :



Binding domain sequence: CAGTTAATAC ACCTAATTAA CAAATCACAC AACGCTTTGT TGTATTGCTG
TTCTAATGTT GTTCCATACA CACTATAACA

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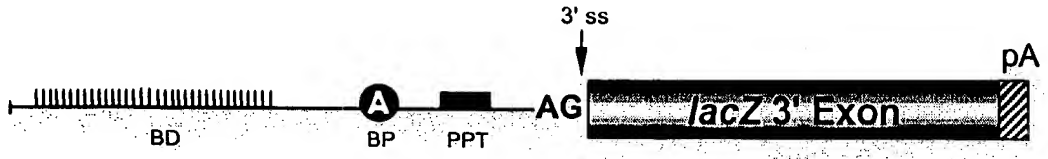
A

HPV-PTM2 with 149 bp binding domain targeted to 3' ss at 409:



Binding domain sequence: CAGTTAATAC ACCTAATTAA CAAATCACAC AACGCTTTGT TGTATTGCTG
TTCTAATGTT GTTCCATACA CACTATAACA ATAATGTCTA TACTCACTAA
TTTTAGAATA AAACTTTAAA CATTTATCAC ATACAGCATA TCGATTCCC

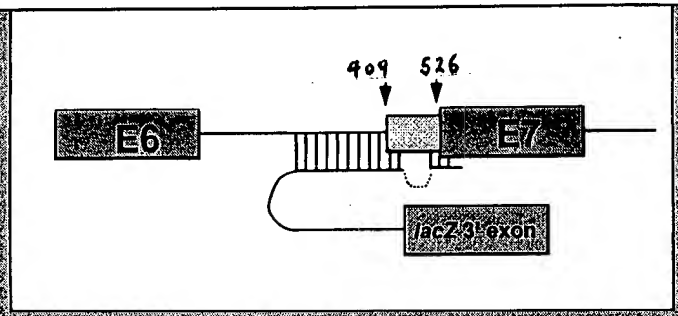
B

FIGURE 52

Binding Domains of HPV-PTM3 and 4

HPV-PTM3 Binding domain (covers both 3' ss at 409 and 526; has 53 bp bubble)

GATGATCTGCAACAAGACATACATCGACCGGTCCA(53 nt bubble)CTTCAGGACACAGTGGCTTTTGAC
AGTTAATACACCTAATTAACAAATCACACAACGGTTTGTTGTATTGCAGTTCTAATGTTGTTCCATACACACTA
TAACAAT



HPV-PTM4 Binding domain (covers both 3' ss at 409 and 526; has 76 bp bubble)

GATGATCTGCAACAAGAC(76 nt bubble)GACACAGTGGCTTTTGACAGTTAATACACCTAATTAACAAATC
ACACAACGGTTTGTTGTATTGCAGTTCTAATGTTGTTCCATACACACTATAACAAT

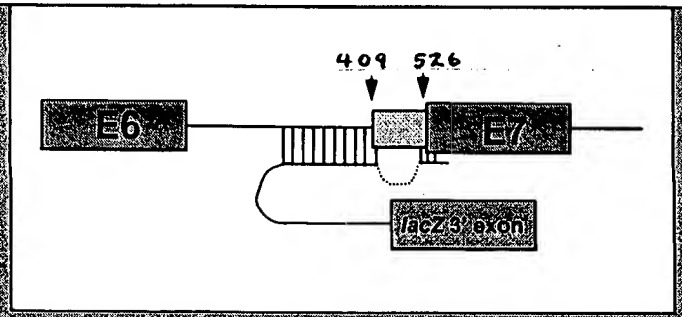


FIGURE 15.3

INTRONN

HPV-PTM5 and 6

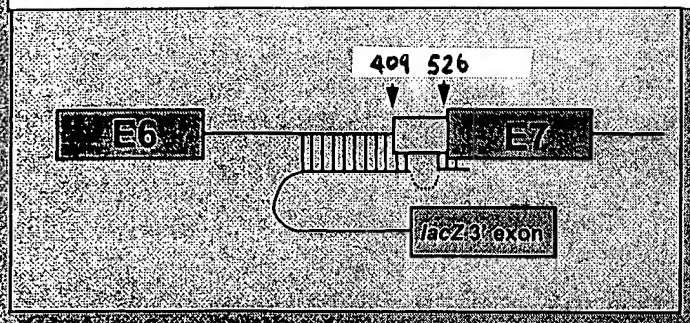
HPV-PTM5, Binding domain (140 nt, has **53 nt bubble**, covers 3'ss at position 409 and 526)

GATGATCTGCAACAAGACATACATCGAC**C**G**G**TCCA.CTTCAGGACACAGTGGCTTTTGACAGTTAATACACCTAATTAACAAATCACACAA**C**G**G**T

TTGTTGTATTGCAGTTCTAATGTTGTTCCATACACACTATAACA

cCCGT

cCCGT

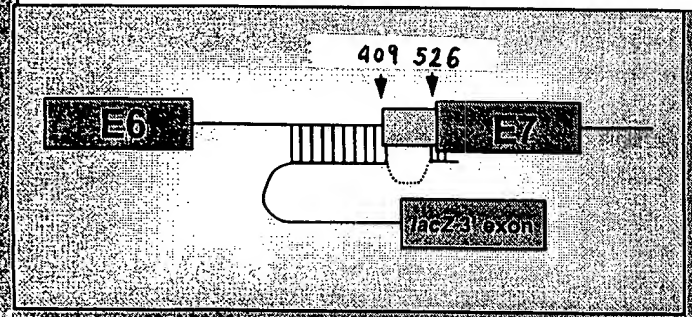


HPV-PTM6, Binding domain (117 nt, has **76 nt bubble**, covers 3'ss at position 409 and 526)

GATGATCTGCAACAAGAC.GACACAGTGGCTTTTGACAGTTAATACACCTAATTAACAAATCACACAA**C**G**G**TTTGTTGTATTGCAGTTCT

AATGTTGTTCCATACACACTATAACA

cCCGT

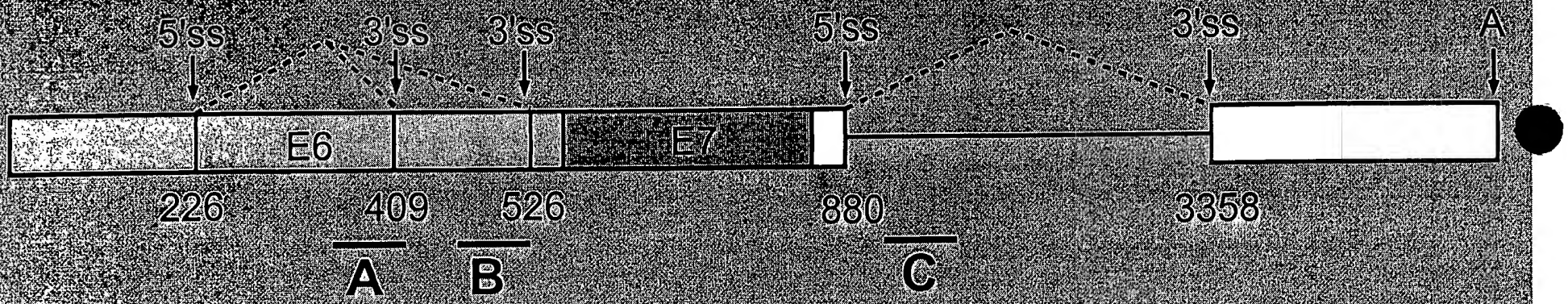


Note: Nucleotides in bold are modified to prevent PTMs cryptic splicing

FIGURE 154

INTRONN

Positions of HPV-PTM Targeting Domains



PTM	Binding Domain Region	Size (nt)
HPV-PTM1	A	80
HPV-PTM2	A	149
HPV-PTM5	A+B	140
HPV-PTM6	A+B	117
HPV-PTM8	C	104
HPV-PTM9	C	174

INTRONN

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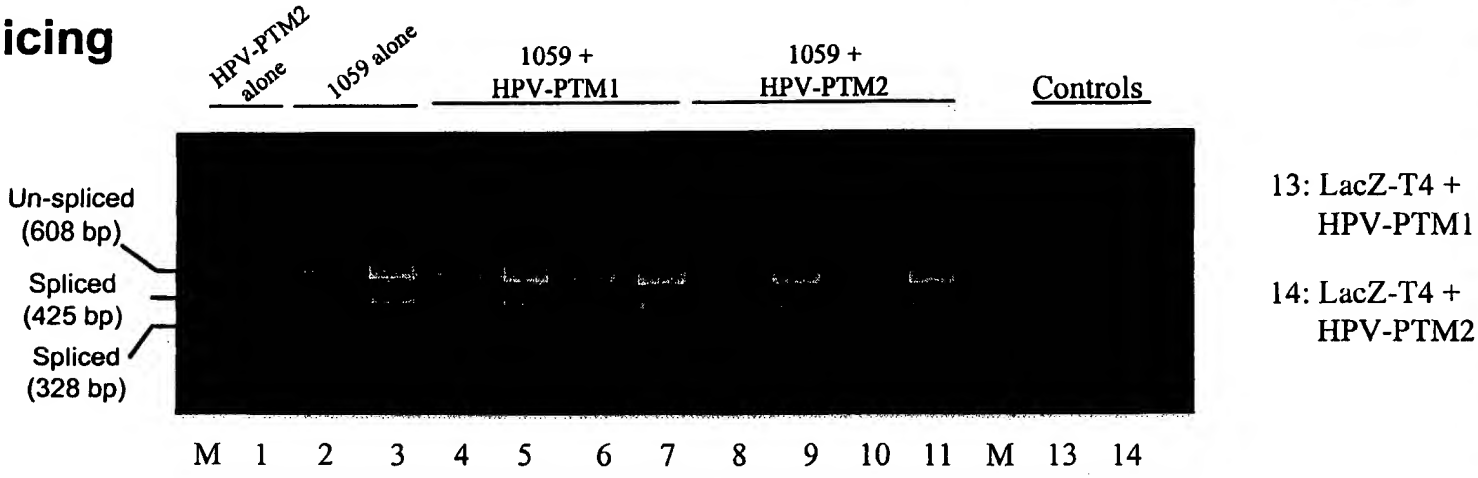
FIGURE 55

Trans-splicing Efficiency of HPV-PTMs in 293T Cells

Cis-splicing



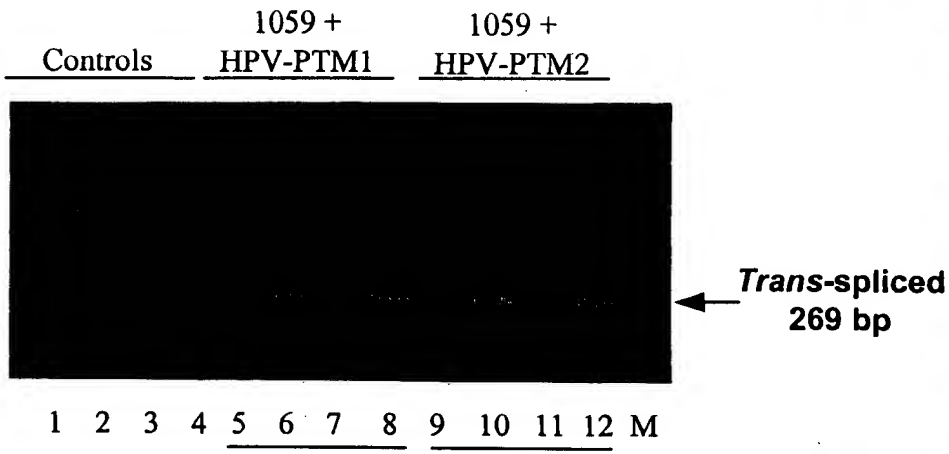
Trans-splicing

- Mock
- HPV-PTM1 alone
- HPV-PTM2 alone
- 1059 target alone

30 cycles

5 – 8: 1059 + HPV-PTM1
9 – 12: 1059 + HPV-PTM2

25 & 30 cycles



RT-PCR Analysis of total RNA

INTRONN

FIGURE 56

Trans-splicing between target pre-mRNA and PTM is accurate (293T cells)

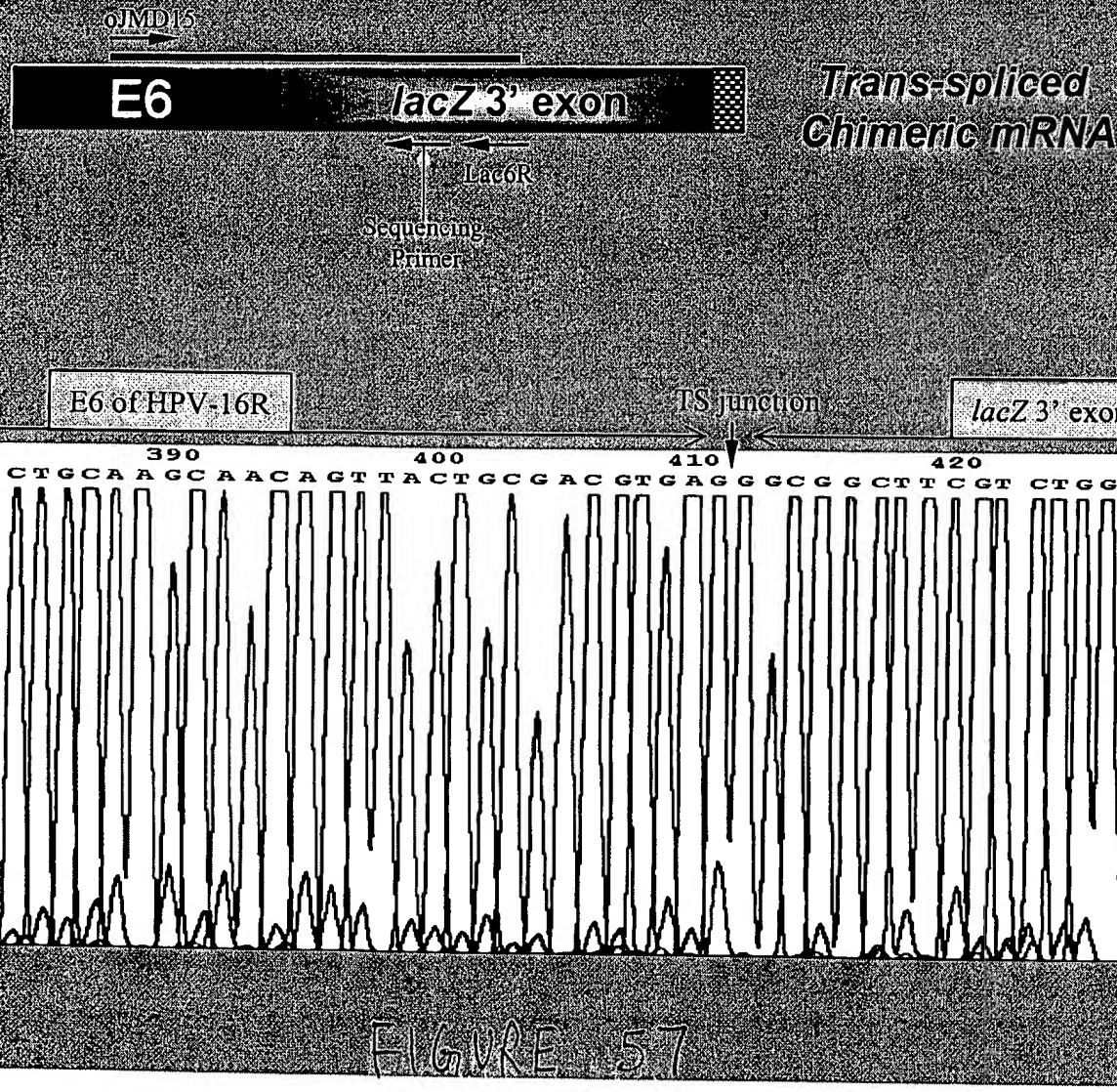
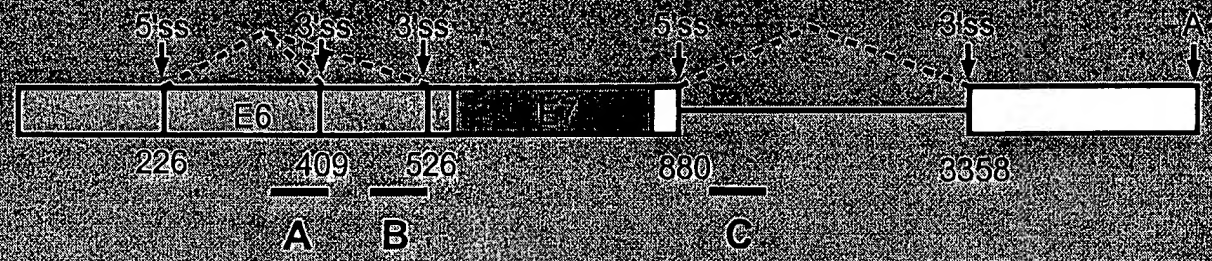


FIGURE 5.7

INTRONN

Trans-splicing in 293 Cells (Co-transfections)

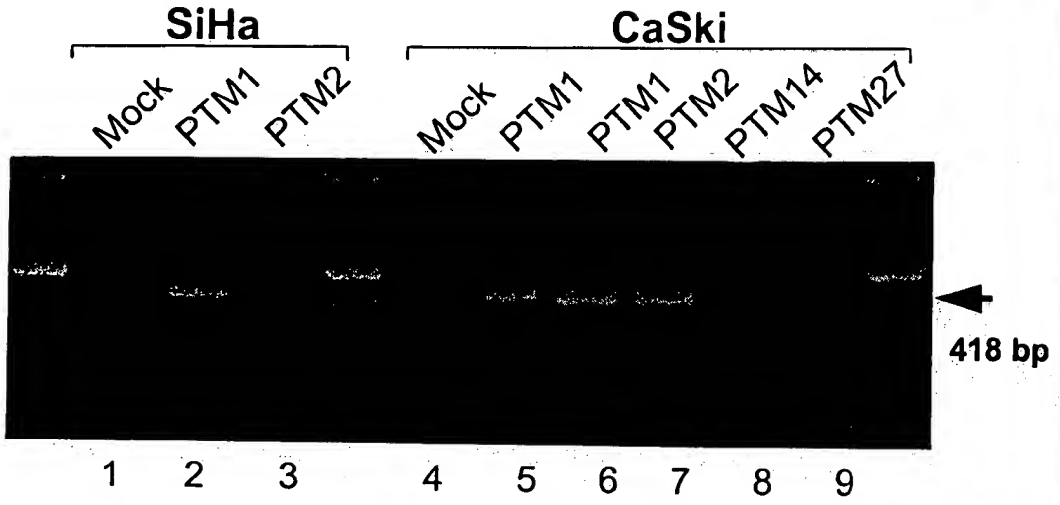


PTM	Binding Domain Region	Size (nt)	% trans-spliced 226 sd	880 sd
HPV-PTM1	A	80	69	0.6
HPV-PTM2	A	149	45	0.9
HPV-PTM5	A+B	140	55	0.8
HPV-PTM5ΔBP/PPT	A+B	140	0.5	0.2
HPV-PTM6	A+B	117	59	1
HPV-PTM8	C	104	7	37
HPV-PTM9	C	174	14	22
CF-PTM27	CF intron	411	0	0

Quantification of *trans*-splicing efficiency using real-time QRT-PCR

FIGURE 58

Trans-splicing into Endogenous HPV Pre-mRNA Target in SiHa & CaSki Cells



RT-PCR Conditions

- Total RNA: 400 ng/rxn
- Primer's: oJMD15 + Lac16R
- # Cycles : 35
- Expected product : 418 bp

SiHa : Single copy
CaSki : ~ 400-500 copy!!

Trans-spliced Chimeric mRNA

Details

- PTM1, PTM2 : HPV targeted, specific
- PTM14 : CF targeted, non-specific, has 23 bp BD
- PTM14 : CF targeted, non-specific, has 411 bp BD

RT-PCR Analysis of total RNA

FIGURE 59

INTRONN

Accurate *Trans*-splicing of HPV-PTM1 in Si Ha Cells (Endogenous target pre-mRNA)

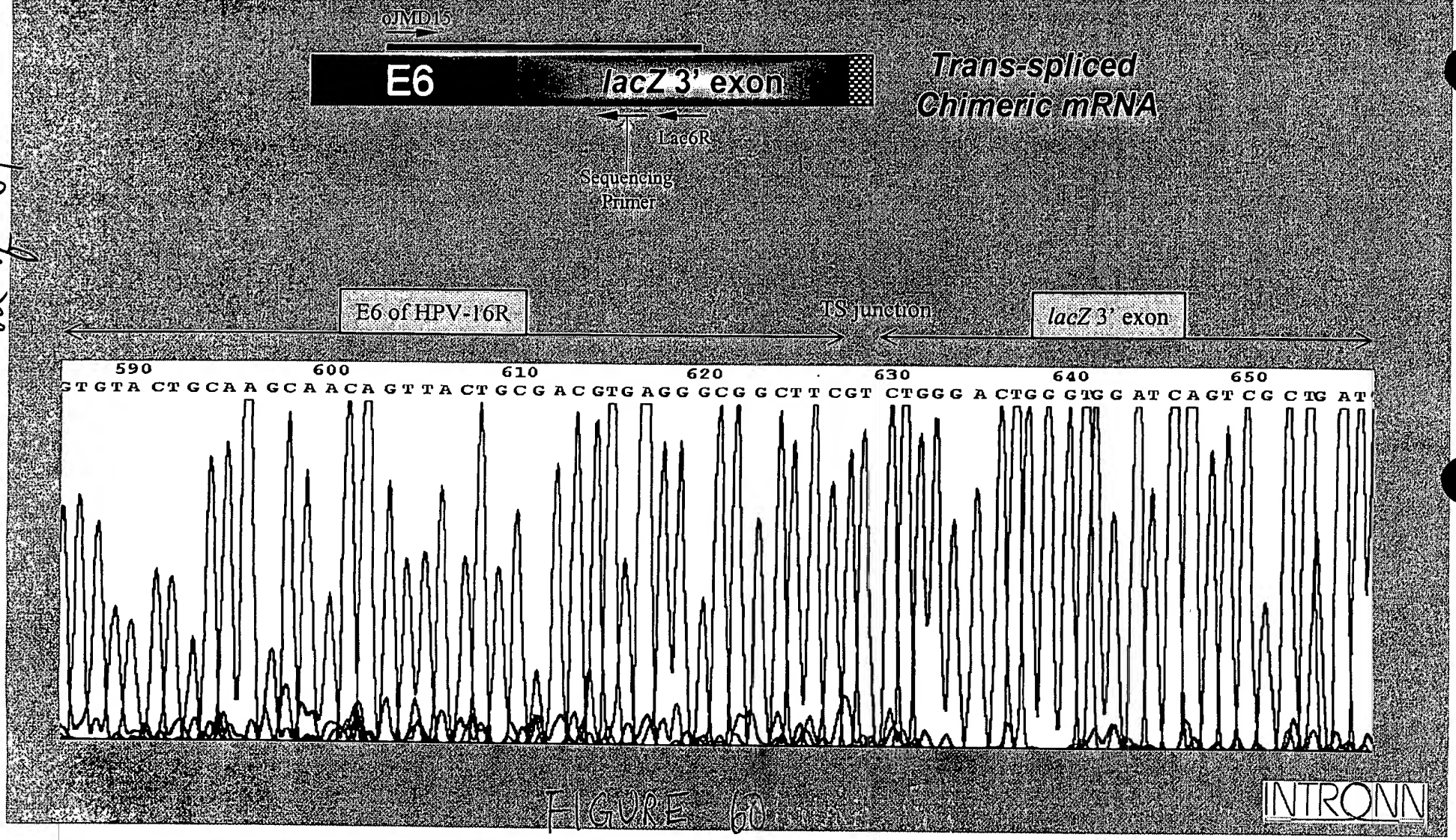


FIGURE 60

INTRONN

Trans-splicing in SiHa Transfections
(Endogenous target)

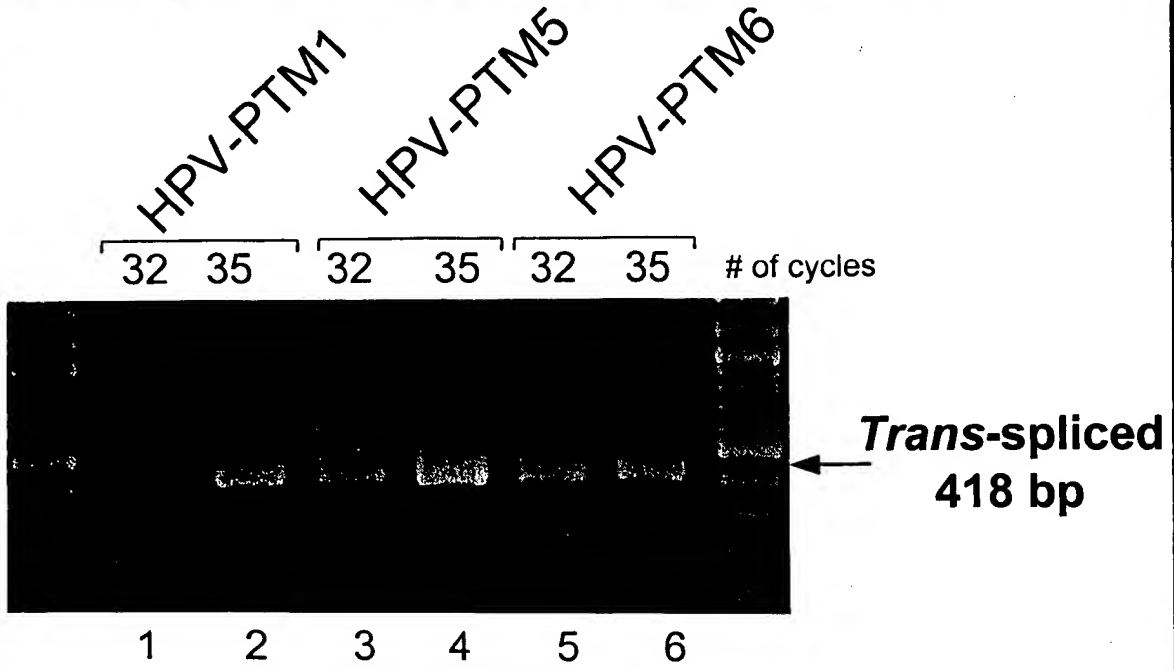
PTM	% *trans*-spliced
pcDNA3.1	0
HPV-PTM1	0.16
HPV-PTM5	0.12
HPV-PTM6	0.11
CF-PTM27	0

Quantification of *trans*-splicing efficiency using real-time QRT-PCR

FIGURE 61

INTRONN

Trans-splicing Efficiency of HPV-PTM1, 5, & 6 in SiHa Cells



***Trans*-spliced 418 bp**

- SiHa cells transfected with 1.5 μg plasmid DNA, LipoPlus
- RNA isolated after 48 hr

- Total RNA: 500 ng/Rxn
- Primers: oJMD15 + Lac16R
- Expected product: 418 bp

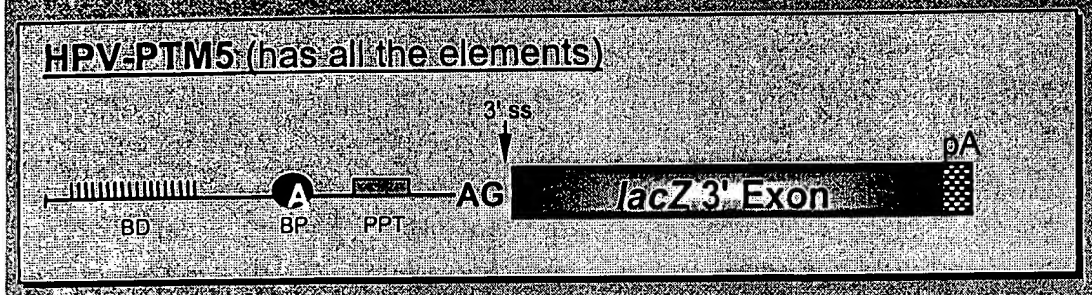
1, 3, 5, : 32 cycles

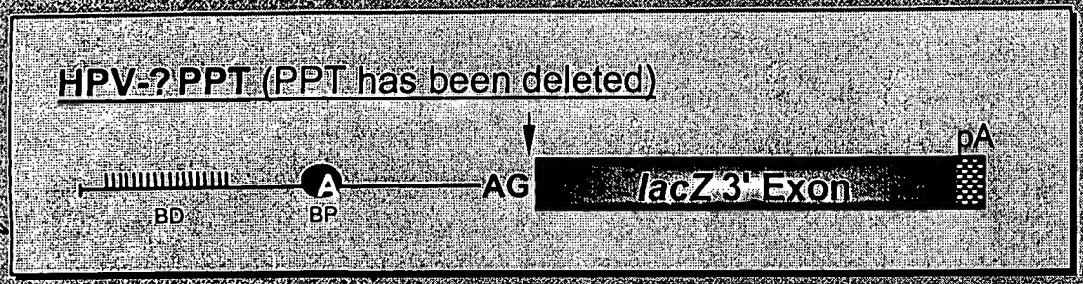
2, 4, 6, : 35

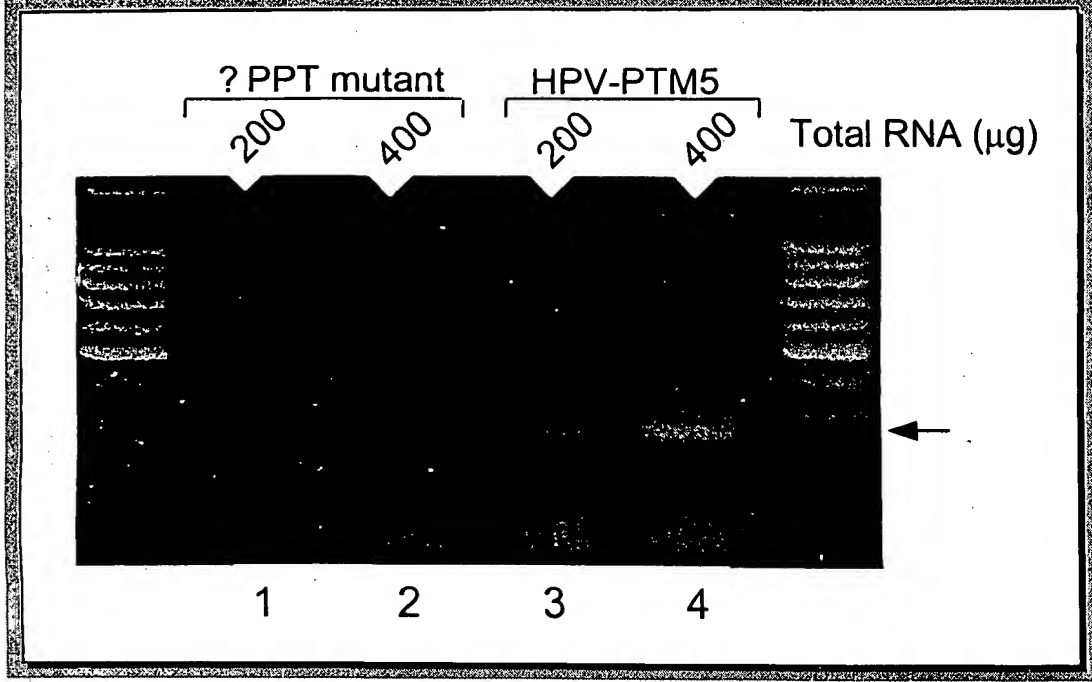
FIGURE 62

INTRONN

Deletion of polypyrimidine tract abolishes *trans*-splicing







Methods:

- SiHa cells transfected with 1.5 μg of plasmid DNA
- Total RNA isolated after 48 hr and analyzed by RT-PCR (30 cycles)

Primers: oJMD15+Lac6R
Expected product: 269 bp

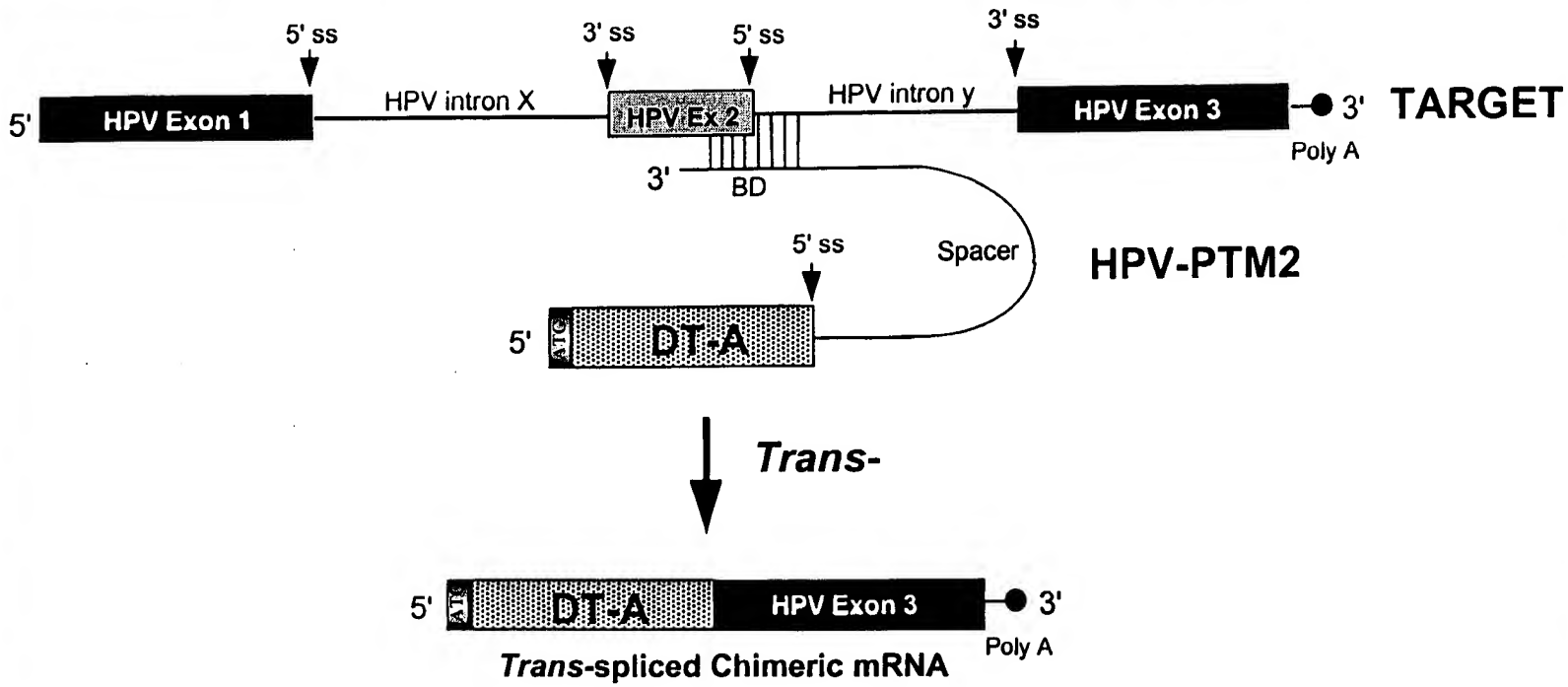
Lanes 1 & 2: RNA from cells transfected with HPV-? PPT (mutant); No trans-splicing detected

Lanes 3 & 4: RNA from cells transfected with HPV-PTM5 plasmid; trans-splicing Detected (269 bp product)

INTRONN

FIGURE 63

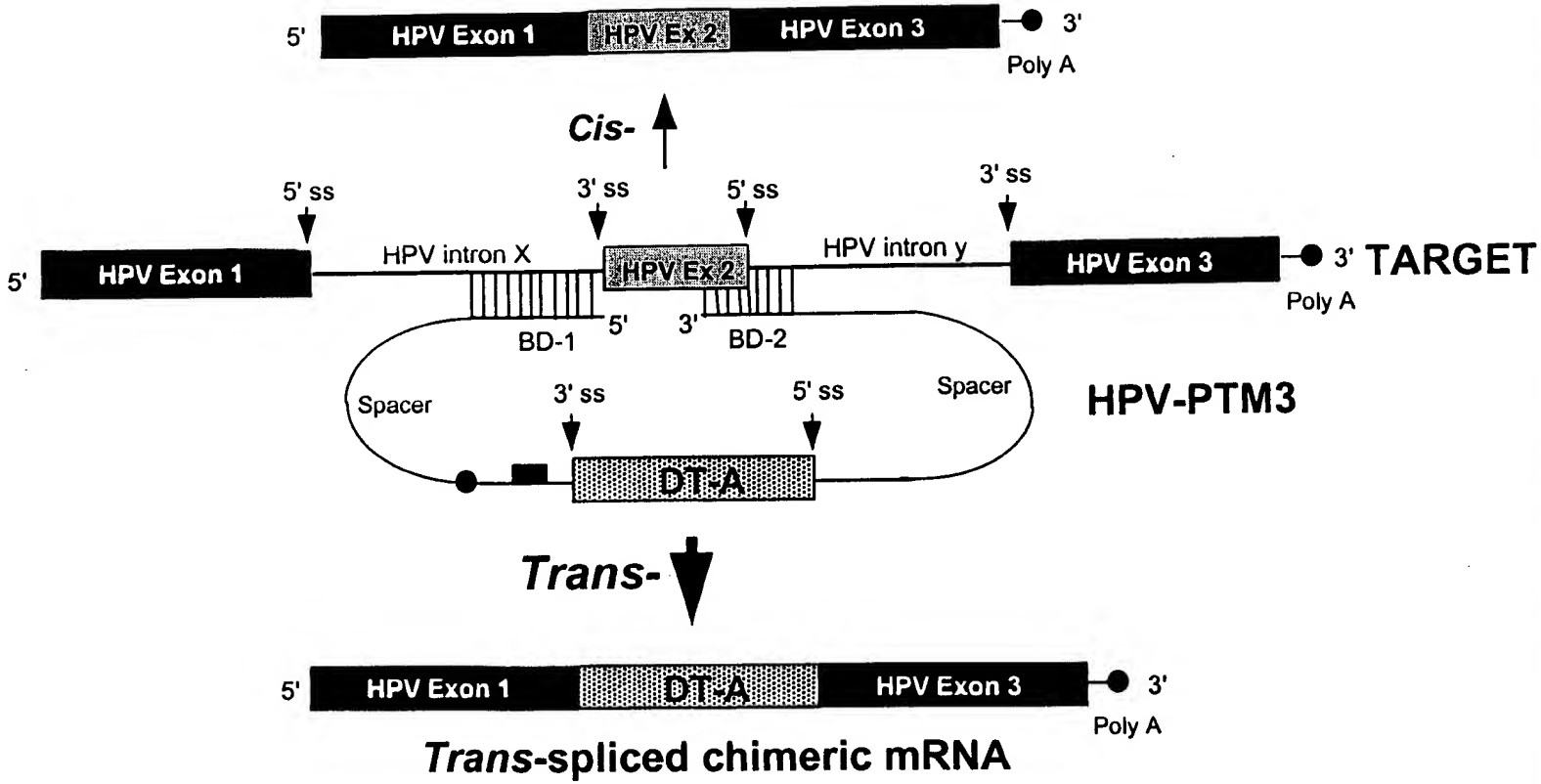
SMaRT Strategy by 5' Exon Replacement



Schematic diagram of a PTM binding to the 5' splice site of the HPV mini-gene target

FIGURE 64

Double *Trans*-splicing



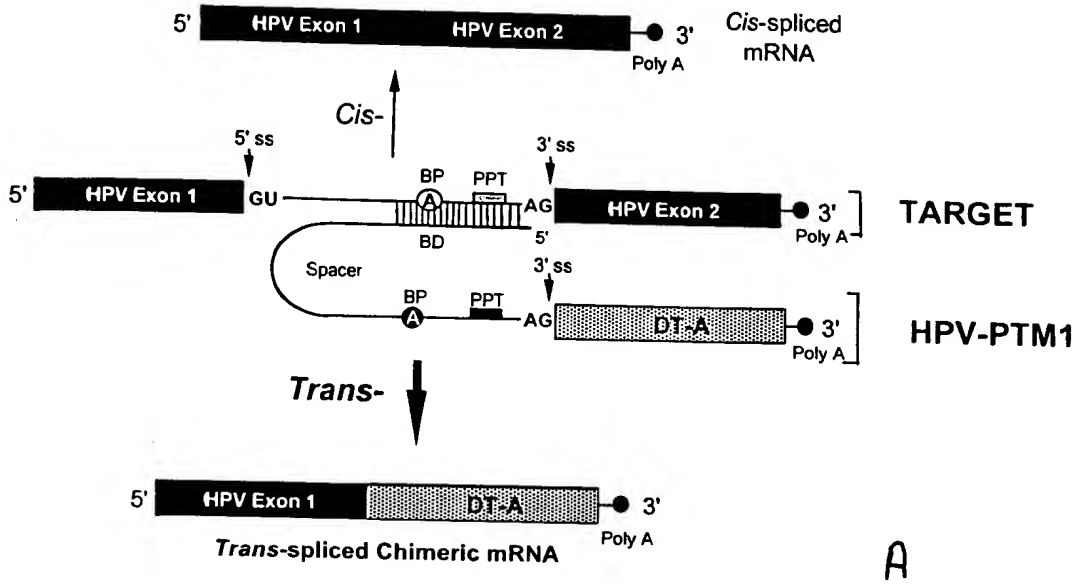
***Trans*-spliced chimeric mRNA**

Schematic diagram of a double *trans*-splicing PTM binding to the 3' and 5' splice sites of the HPV mini-gene target

INTRONN

FIGURE 65

SMaRT Strategy by 3' Exon Replacement: **Schematic diagram of a PTM binding to the 3' splice site of the HPV mini-gene target**



SMaRT Strategy by 5' Exon Replacement: **Schematic diagram of a PTM binding to the 5' splice site of the HPV mini-gene target**

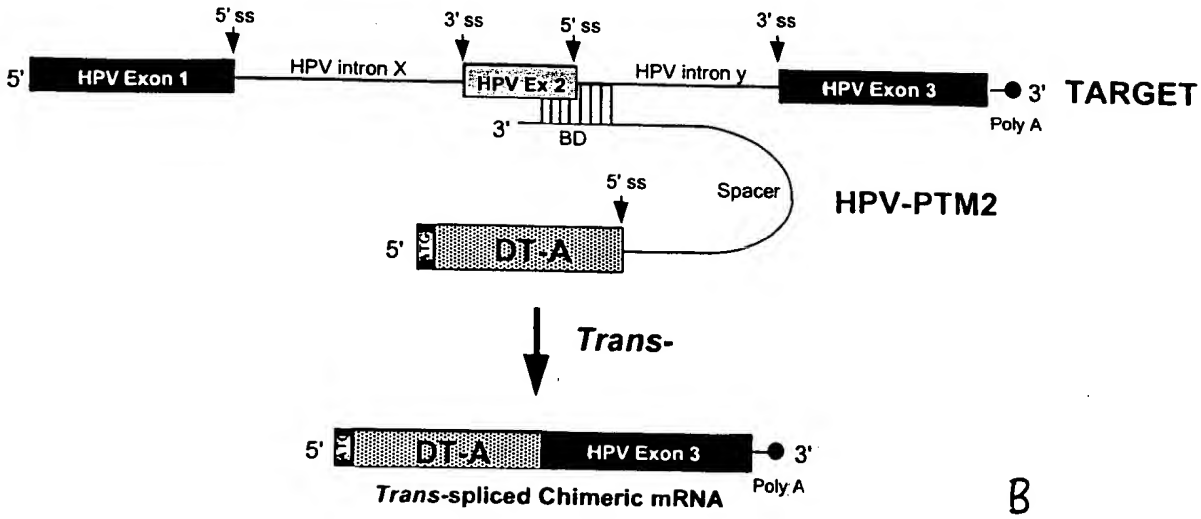


FIGURE 66

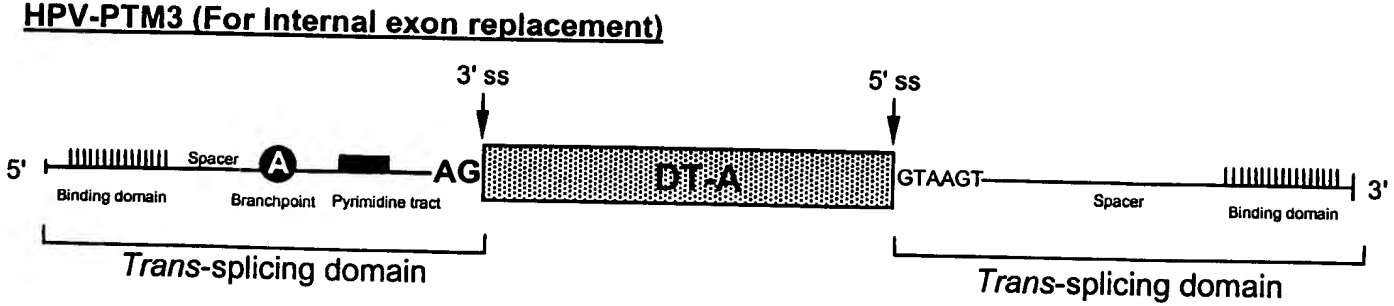


FIGURE 67